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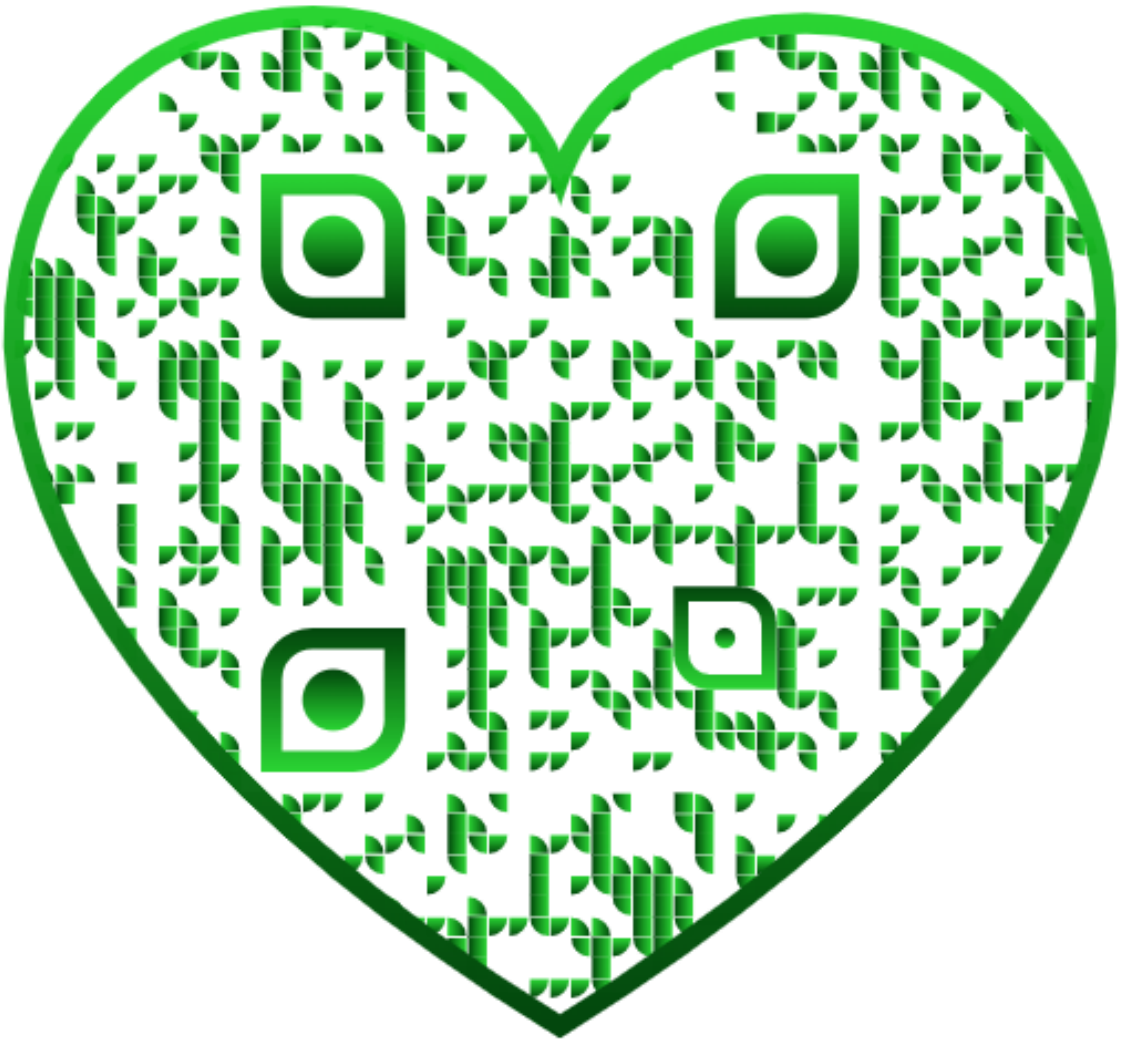

Eric Lichtfouse
Editor

Organic Farming, Pest Control and Remediation of Soil Pollutants

Springer

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Sustainable Agriculture as a Central Science to Solve Global Society Issues

Eric Lichtfouse

Nature does not hurry, yet everything is accomplished.
Lao Tzu

Abstract Serious global issues such as poverty, illness, food prices, climate changes, global market, pollution, pest adaptation and resistance, soil degradation, decreasing biodiversity and desertification can be explained by the increasing artificialization of human society. Since most issues are now intertwined they cannot be solved anymore by the classical fireman approach. In that respect, the structure of actual science and governmental institutions are probably outdated and should evolve to meet global challenges. Unexpectedly, agronomy appears as a central science to solve current societal issues because agronomists are trained to manage the input of many disciplines such as plant biology, soil science, climate sciences, ecology and chemistry.

Keywords sustainable agriculture · global society issues · artificialization · agronomy

Starving people in poor nations, ill and fat people in rich nations, increasing food prices, climate changes, increasing fuel and transportation costs, flaws of the global market, worldwide pesticide pollution, pest adaptation and resistance, loss of soil fertility and organic carbon, soil erosion, decreasing biodiversity, and desertification are current acute problems that threatens our planet. Most current human issues can be explained by the "artificialization" of society (Fig. 1).

Artificialization began in ancient times with the start of one of the oldest human practices—agriculture. The beginnings of wheat domestication can be traced back to about 8,000 years BC (Araus et al., 2007). At that time, humans switched progressively from a nomad life involving hunting animals and eating wild plants and

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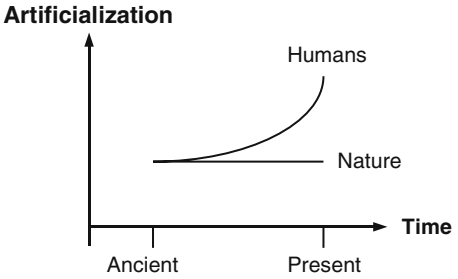

Fig. 1 Most current issues of society can be explained by the increasing artificialization of human behavior. The artificialization started to increase fast during the industrial revolution, leading to global issues.

fruits—typical of animal behavior—to a more settled life of growing crops, harvesting, and storing food. Tribes rapidly invented money, in the form of shells or salt at first, as a means to exchange goods with other tribes. That was the beginning of the financial market. Tribes also invented territories and borders. That was the beginning of nations. Early social behaviors already had benefits such as a secure stock of food for winter, as well as drawbacks such as wars to conquer wealthier territories. For quite a long time, until the start of the industrial revolution around 1850, the negative impacts on society were limited and local because social groups and nations were independent and small. Negative impacts on society were also restricted by time because the transport of food, goods, and humans was slow and mainly local.

Artificialization increased dramatically during the industrial revolution. The advent of motor boats, cars, and planes allowed rapid transportation of goods over long distances. Social groups and nations began to be less and less independent and began to rely on other countries for food and goods. This worldwide behavior led to tremendous benefits such as medicine that has significantly increased the human lifetime. But this also led to worldwide negative consequences such as World Wars I and II, Chernobyl, and more recently, climate change, poorer countries, and ill and fat wealthy people.

Until now, societal issues have been solved primarily by using the "fireman" approach—or the "pain-killer" approach as a more medical version—by which an individual problem is solved by an individual solution. Such an approach does not work anymore for two reasons, at least. First, all systems, mechanisms, and activities are closely intertwined. For instance, food production is closely linked to health, climate change, transportation, market, finance, and politics. Therefore, applying a remedy to only one element of this system will not work because the remedy will induce negative impacts on other elements in the end. Only solutions that consider the whole system and its connections will have a chance to succeed now. Second, the fireman approach does not treat the source of the problem. It only treats the negative consequences. Here, an obvious solution is to identify the problem sources and anticipate potential future problems.

From those observations, two pieces of advice can be given. First, actual borders, names, and fields covered that separate government institutions are probably outdated. For example, an agriculture department should not be separated from health, economics, or transportation departments because most agricultural issues are linked to health, economics, and transportation. The name, structure, mechanisms and fields covered by departments should thus evolve to take into account the sources and connections of modern issues. Second, in a similar way, the division of sciences into disciplines such as physics, biology, and chemistry is not in line with actual scientific issues that are solved by the input of several disciplines. The names, structures, mechanisms, and fields covered by scientific disciplines should thus evolve to adapt into actual, interconnected scientific issues.

For a long time, agronomy has been considered a soft "side" science because food production was not really an issue in rich nations after the start of industrial farming around 1960. Now, unexpectedly, agronomists appear as the best scientists to solve current societal issues of food, climate change, health, and poverty. Indeed, agronomists are typically used to solve issues that need the input of many sciences, such as plant biology, soil science, climate sciences, environmental chemistry (Lichtfouse et al., 2005), geology, sociology, and economics (Lichtfouse et al., 2004, 2009). They also work on very complex research objects, the behavior of which is seldom reproducible. Sustainable agriculture thus now appears to be a central science. Sustainable agriculture is thus the best fitted science to solve current issues, to anticipate future negative impacts, and to define novel practices that will make the world safer for our children.

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Mother of Necessity: The Soil

Rattan Lal

Abstract Almost all the increase in future population will occur in developing countries where soil and water resources are already under great stress. The pressure on finite soil resources is likely to be exacerbated by global warming, soil degradation, pollution and decrease of available fresh water, urbanization and industrialisation, and increasing price of fertilisers. Here I describe five tenets of sustainable agriculture to improve crop yields and soil resources. The tenets are (1) enhancement of soil organic carbon and soil structure, (2) creating a positive nutrient budget, (3) soil restoration, (4) adapting agriculture to climate change, and (5) land saving technologies.

Keywords soil erosion · climate change · world popultion · water · fertilizer · crop yield

The world population of 6.75 billion in 2008 is projected to reach 9.2 billion by 2050. Almost all the increase in future population will occur in developing countries where soil and water resources are already under great stress. Between 2008 and 2050, regional population is projected to increase from 827 to 1,761 million inhabitants in sub-Saharan Africa (+ 113%), from 364 to 595 million in the Middle East and North Africa (+ 63%), from 35 to 45 million in Oceania (+ 41%), from 579 to 769 million in Latin America and the Caribbean (+ 33%), from 342 to 445 million in North America (+ 30%), and from 3,872 to 4,909 million in Asia (+ 27%). These trends in regional population increases contrast with that of projected declines in the population of Europe from 731 million in 2008 to 664 million in 2050 (–9%), and an overall increase in world population from 6,750 million in 2008 to 9,191 million in 2050 (+ 36%).

The pressure on finite soil resources for meeting the demands of the increase in population for food, feed, fiber, and fuel is likely to be exacerbated by several inter-

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active factors. Notable among these factors are: (1) global warming, (2) soil degradation, (3) decline in fresh water supply along with pollution and contamination of water resources, (4) urban encroachment and industrialization, and (5) decrease in use efficiency and increase in price of energy-based input such as fertilizer and irrigation water.

Despite the challenges, there is a vast scope for enhancing yields of food crops in developing countries. The national yields of food crop staples in Ethiopia, India, and the developed countries, for example, is 1,870, 3,284, and 6,810 kg/ha for rice; 1,469, 2,601, and 3,110 kg/ha for wheat; 2,006, 1,907, and 8,340 kg/ha for corn; 1,455, 797, and 3,910 kg/ha for sorghum; 730, 332, and 1790 kg/ha for cowpeas; and 1,026, 814, and 7,980 kg/ha for chick peas, respectively.

There is a strong need to alleviate biophysical and socioeconomic constraints to increase agronomic productivity in the developing countries of Asia and Africa. The *maximum yield potential* of an ecoregion is determined by climatic factors—e.g., solar radiation, soil and air temperatures, and evapotranspiration—because there are no soil or plant-related constraints. In comparison, the *on-station crop yield*, through adoption of recommended management practices, is determined by a range of factors including drought stress, low soil fertility, farming system, and soil-water management options. The *attainable crop yield* is governed by social, economic, and institutional factors such as land tenure, market and infrastructure, and support services, etc. The *actual farm yield* is affected by the prevalent farming system,

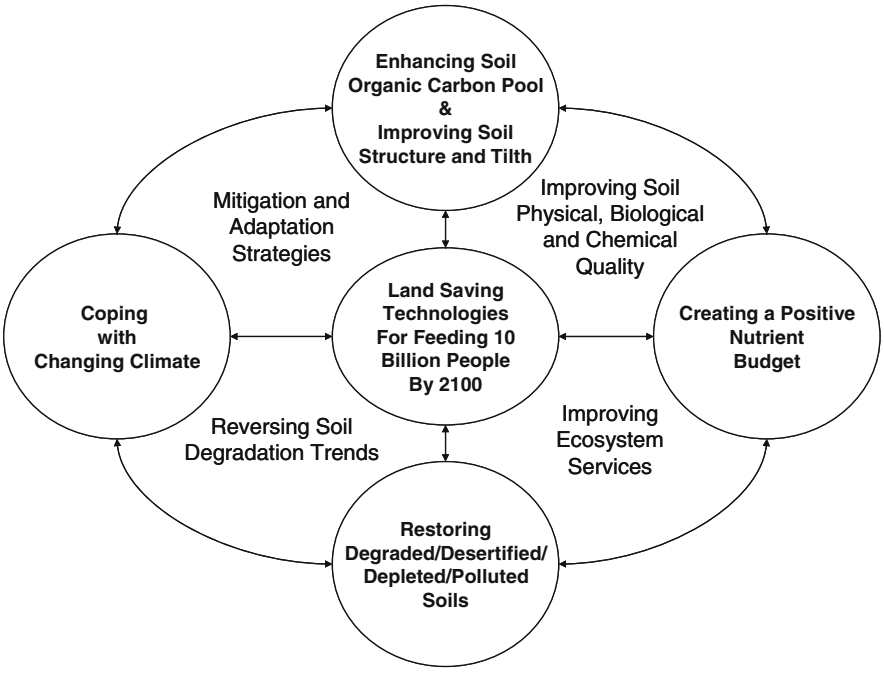
Enhancing Soil Organic Carbon Pool & Improving Soil Structure and Tilth
Mitigation and Adaptation Strategies
Improving Soil Physical, Biological and Chemical Quality
Coping with Changing Climate
Land Saving Technologies For Feeding 10 Billion People By 2100
Creating a Positive Nutrient Budget
Reversing Soil Degradation Trends
Improving Ecosystem Services
Restoring Degraded/Desertified/ Depleted/Polluted Soils

Fig. 1 Five tenets of sustainable agriculture

the type and severity of soil degradation, soil evaporation, and losses of water and nutrients from runoff and soil erosion.

There is a large "yield gap" between the "on-farm" yield and "maximum yield potential" in developing countries. Bridging the yield gap necessitates the adoption of recommended practices for sustainable management of soil resources. Recommended practices are based on the five tenets of sustainable agriculture, as follows (see Fig. 1):

1 Enhancement of Soil Organic Carbon and Soil Structure

It is essential to enhance organic carbon levels above the threshold minimum of 1.2% in the root zone for the major soils of the tropics. Increasing organic carbon concentration implies maintaining a positive ecosystem C budget. There also exists a close relationship between organic carbon concentration and soil structure as moderated by the activity and species diversity of soil fauna. Increase in organic carbon, and the attendant improvement in structure and tilth, enhance water and nutrient storage and availability, and increase soil aeration. Improving the overall soil physical quality is essential to increasing root growth and development, and enhancing uptake of water and nutrients.

2 Creating a Positive Nutrient Budget

Most cropland soils of sub-Saharan Africa and South Asia have experienced a negative nutrient budget of 30–40 kg of NPK/ha/yr on a continental scale since the 1960s. The negative nutrient budget is caused by the prevalence of extractive farming practices, including removal of crop residues for fodder and fuel, and the use of animal dung for household cooking, low or no application of chemical fertilizers, and unbalanced application of nutrients—e.g., N rather than P, and K because of subsidies for N. Soils depleted of inherent nutrient reserves do not respond to other inputs such as the adoption of improved varieties.

3 Soil Restoration

Restoring the quality of degraded, desertified, depleted, and contaminated soils is essential for meeting the demands of an increasing population with growing aspirations for high standards of living. In addition to enhancing the net primary productivity and agronomic output, restoring degraded and desertified soils also improves the environment, especially in relation to water quality through the decline in transport of dissolved and suspended loads and the attendant reduction in nonpoint source pollution; and in relation to air quality through a decline in wind erosion, reduction in emission of trace gases and aerosols, a decline in efflux of particulate material, and improvements in biodiversity. Soil restoration is also closely linked with climate change through the impact on the global C cycle.

4 Adapting Agriculture to Changing Climate

Reducing the adverse impact of climate change necessitates adapting agronomic practices to spreading risks. Ex-ante risk management options include: (1) soil management by mulching, no-till farming, delayed fertilizer application, and integrated nutrient management options, runoff management and adequate weed control; (2) plant management involving variety selection, staggered time of planting, low-planting density, bunch planting, intercropping, and (3) farming system management involving diversification, agroforestry, and mixed farming.

5 Land Saving Technologies

With the decreasing availability of per capita land area and supply of fresh water resources for agriculture, it is essential to increase productivity per unit area of existing land through agricultural intensification. The goal is to intensively cultivate the best soils using best management practices to obtain the best yield so that land can be saved for nature conservancy. With the adoption of specialized technologies that builds upon traditional knowledge but also use modern scientific innovations, per capita land area requirements can be decreased to less than 0.03 ha. With10 billion people by 2100, this would still amount to 3 billion ha of cropland compared with the present cultivated land area of 1.5 billion ha.

Implementing these five tenets is an essential prerequisite to enhancing the respectability of the farming profession (Fig. 1). Resource-poor farmers in developing countries are at the lowest level of society. The profession practiced by small-size landholders involves drudgery and substandard living. Replacing the back-breaking hoe and breaking the poverty trap are essential to improving the standard of living.

Soil—the essence of all terrestrial life—embodies Mother Nature in that it has the capacity to meet all human needs without the greed. This capacity must be sustained, improved, and restored through a paradigm shift. This shift implies that (1) the soil surface must be protected against erosion by water and wind and prevent loss of water, carbon and nutrients out of the ecosystem; (2) elemental cycling must be strengthened by soil application of biosolids; (3) activity and species diversity of soil fauna must be promoted; (4) soil must become a sink of atmospheric CO_2 and CH_4 to reduce emission of other greenhouse gases (e.g., N_2O); and (5) soil's ability to denature and filter pollutants as a biomembrane must be improved.

The conceptual paradigm shift necessitates the widespread adoption of recommended management practices. Important among these are: (1) no-till farming with residue mulch, cover cropping, and manuring; (2) water harvesting, recycling, and conserving in the root zone for improving productivity per unit consumption of fresh water and utilizing the rainfall more effectively; (3) integrated nutrient management involving combination of biological N fixation, recycling, and judicious use of chemical fertilizers and amendments; (4) building upon traditional knowledge and using modern technologies while promoting farmer innovations; and (5) improving channels of communication and networking.

Successful implementation of these technologies implies involving farmers in the decision-making process at all stages in identifying the priorities of research and practice. While sustainable management of soil is the mother of the necessity, its implementation is in the hands of the farmer. The goal is to make “soil and farmer” at the center of the action, to successfully meet the challenges of changing climate and human needs and aspirations.

Technology Without Wisdom

Rattan Lal

Abstract Despite impressive increases of crop yields during the second half of the 20th century, several environmental concerns such as water contamination by pesticides, accelerated soil erosion, biodiversity reduction, and emission of greenhouse gases (CO_2, CH_4, N_2O) are attributed to agriculture intensification. The world population of 6.5 billion now and 10 billion by the end of the 21st century must be fed, degraded soils must be restored, quality and quantity of fresh water must be enhanced, biodiversity must be increased, the global warming must be brought under control, and human equity must be achieved. It is heartening to realize that the answers to these questions lie in judicious and sustainable management of world's soils. However, success in this mission will depend on our ability to learn from the past mistakes. The problem is not with the technology, but with its abuse. It was over fertilization, overuse of pesticides, excessive application and use of poor quality of water and free electricity for pumping the irrigation water, excessive and unnecessary plowing of fragile soils on sloping terrains, landforming with bulldozers and construction equipment, extractive farming practices that create negative nutrient balance, and soil mining for brick making that have caused the problems. Soil scientists have the knowhow to make the desert bloom. Important technological innovations to achieve this include no-till farming with residue mulch and cover crops, judicious use of fertilizers and integrated nutrient managements, precision farming to meet soil specific needs, water harvesting and recycling along with drip irrigation, retiring marginal lands for nature conservancy and restoring wetlands, and using integrated watershed management approaches to improve water resources. Misusing any technology is a blunder that the world cannot afford, not anymore. Nine blunders of humanity are listed, including seven identified earlier by Mahatma Gandhi.

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Keywords Soil degradation · Pesticide pollution · Water · Biodiversity · Technology

Despite the impressive gains made in achieving high crop yields during the second half of the 20th century, several environmental concerns are attributed to the expansion and intensification of agriculture. Notable among these are contamination of natural waters and air pollution attributed to the use of fertilizers and pesticides, accelerated soil erosion, and sedimentation of waterways and reservoirs attributed to unnecessary and excessive plowing, non-point source pollution, and eutrophication of natural waters attributed to the transport of dissolved and suspended loads from agricultural lands, reduction in biodiversity caused by monocropping, and emission of greenhouse gases (CO_2, CH_4, N_2O) caused by the mineralization of soil organic matter, raising of livestock, cultivation of rice paddies, and application of nitrogenous fertilizers.

These concerns have been raised in several well-known books since the 1930's, including "The Grapes of Wrath" by John Steinbeck (1939), "Rape of Earth" by G. V. Jacks (1939), "Plowman's Folly" by Edward Faulkner (1942), "The Groundnut Affair" by A. Wood (1950), "Soil and Civilization" by E. Hyams (1952), "Silent Spring" by Rachel Carson (1962), and "The Closing Circle" by Barry Commoner (1972). There have been similar books since the 1990s and early 2000s including "Collapse: How Societies Choose to Fail or Succeed" by Jarred Diamond (2004), "Our Ecological Footprint" by M. Wackernagel and W. E. Rees (1996), "Soils and Societies" by J. R. McNeill and V. Winiwaiter (2006), and "The Revenge of Gaia" by James Lovelock (2006). The book "The Violence of Green Revolution" by Vandana Shiva (1991) warned about the social and human dimensions of the Green Revolution. These warnings are fully justified because contaminating the environment, polluting the atmosphere, degrading soils, causing extinction of species, and creating social and gender disparity are not acceptable regardless of the reason. Such reality checks are essential to keeping the scientific community focused and helping to keep their feet squarely on the ground (soil).

As soil scientists, one of our professional goals is to develop and promote the adoption of soil, water, and crop management technology, which upholds the premise that access to adequate and nutritious food and clean living environments are two basic human rights that must be respected. That being the goal, the achievements of the Green Revolution of the 1960s and 1970s in enhancing food production and averting mass starvation of billions of people are fully justified and a true success story. Furthermore, the successes achieved in Asia must also be repeated in sub-Saharan Africa. The latter implies that the use of fertilizers, pesticides, and irrigation will need to be increased, especially in sub-Saharan Africa. Therefore, the pertinent question that needs to be objectively and critically addressed is: How can food production be enhanced and hunger/malnutrition eliminated while improving the environment? While this is a tall order, there is no choice. The world population of 6.5 billion now and 10 billion by the end of the 21st century must be fed, degraded/desertified soils must be restored, quality and quantity of fresh water must be enhanced, biodiversity must be increased,

global warming must be brought under control, and social/gender/ethnic equity must be achieved.

It is heartening to realize that the answers to these questions, difficult and complex as they may be, lie in judicious and sustainable management of the world's soils. With that premise, soil scientists have a great opportunity to rise to the occasion and meet the challenge. However, success in this mission will depend on our ability to learn from past mistakes. Thus, it is important to identify the causes of the adverse effects of adopting Green Revolution technology on soil, water, biodiversity, climate, and the general social fabric.

A critical examination of the cause-effect relationship indicates that "the problem is not with the technology, but with its abuse. It was over-fertilization, overuse of pesticides, excessive application and use of poor water quality and free electricity for pumping the irrigation water, excessive and unnecessary plowing of fragile soils on sloping terrains, landforming with bulldozers and construction equipment, extractive farming practices that create negative nutrient balances, and soil mining for brick-making that have caused the problems". Just as nuclear energy can be used either for generating power and curing disease, or for destruction of nature and humanity, so does the management of soil and natural resources impact how we choose to use the technology.

Soil and environmental degradation are indicative of the degree of the societal care of the soil. As Lowdermilk (1939) wrote, "Individuals, nations and civilizations write their records on the land—a record that is easy to read by those who understand the simple language of the land." Aldai E. Stevenson (1952) wrote, "Nature is neutral. Man has wrested from nature the power to make the world desert or to make the desert bloom." All environmental issues (e.g., soil degradation, desertification, global warming) are a creation of human misadventures with nature.

Soil scientists have the know-how to make a desert bloom. Important technological innovations that can achieve this include no-till farming with residue mulch and cover crops, judicious use of fertilizers and integrated nutrient managements (INM), precision farming to meet soil-specific needs, water harvesting and recycling along with drip irrigation/fertigation, retiring marginal lands for nature conservancy and restoring wetlands, and using integrated watershed management approaches to improve water resources. In addition to adopting strategies of sustainable management of soil and water resources, use of integrated pest management (IPM) in conjunction with biotechnology and transgenic plants are also critical to transforming traditional agriculture in developing countries. Indeed, the appropriate use of biotechnology can facilitate the development of new genotypes with high-productive potential under biotic and abiotic stresses. Since the evolution of life on Earth, Mother Nature has always transformed species to promote and facilitate the adaptation of plants and animals to specific ecological niches. As ancient Sanskrit scriptures stated during 1500–2500 B.C., "One life form changes into another till it reaches perfection, and there are 8.4 million life forms." So, working with nature in developing plants that can better withstand biotic and abiotic stresses is appropriate, beneficial, and in accord with natural processes. Why not?

Misusing any technology is a blunder that the world cannot afford—not anymore. Mahatma Gandhi listed seven blunders of humanity. These include: (1) wealth without work, (2) pleasure without conscience, (3) commerce without morality, (4) worship without sacrifice, (5) politics without principles, (6) knowledge without character, and (7) science without humanity. It is probable that if Gandhi were alive today, he might have increased the list by adding two more: (i) Education without relevance, and (ii) technology without wisdom.

Transgenic Cotton for Sustainable Pest Management: A Review

Jorge B. Torres, John R. Ruberson and Mary Whitehouse

Abstract Transgenic cotton has significantly altered pest control in this crop during the last decade. Cotton was one of the first widely cultivated *Bacillus thuringiensis* (Bt) insect-resistant and herbicide-tolerant (Ht) transgenic plants. Over 300 transgenic cotton varieties expressing single or dual Bt proteins targeting lepidopteron larvae, as well as pyramided varieties with herbicide tolerance, are available to growers. Potential negative impacts of transgenic plants, however, have generated concerns over deploying these plants over extensive crop areas, such as those occupied by cotton. Nearly 8% of 33.8 million hectares has been cultivated with Bt cotton with the trend to increase in future seasons. Hence, weediness, gene flow, and impact on nontarget organisms by Bt and Ht cotton have been closely studied during the past decade. Despite justifiable concerns over potential risks, the data show neither a significant negative impact nor the development of field resistance by cotton pests. Results of nontarget impact registered four negative impacts on natural enemies, which are discussed here. No weediness and gene flow have been shown in over 333 published results, although little data exist for the risks of gene flow. Regarding insect resistance, several factors underline resistance appearance in field population, including species biology and interactions with the environmental conditions population. Modeling of the evolution of resistance in a field population to Bt proteins has been conducted and the use of single or dual Bt protein varieties might reach some failure due to resistance depending on gene frequency-conferring resistance in the population. Planting transgenic cotton, therefore, requires effort and vigilance to ensure sustainability of the system, including the planting of mandatory refuges and monitoring insect and weed resistance. This article presents and discusses seven sections beyond an introductory section: What is a transgenic plant, conventional and transgenic plant breeding methods in insect-resistant cotton, how transgenic cottons were developed (Bollgard®, WideStrike®, VipCot® and herbicide tolerant cottons), potential nontarget effects of Bt cottons, resistance and resistance management, Bt cotton perspective in Brazil, and the future of transgenic and pest management in cotton.

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Keywords Bt cotton · Integrated pest management (IPM) · Herbicide-tolerant cotton · Nontarget impact · Sustainability

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Abbreviations

2,4-D	2,4-dichloro-phenoxyacetic acid
aad	Aminoglycosidase adenyltransferase
aadA	Streptomycin adenyltransferase
aph4	Hygromycin-B phosphotransferase
bar	Bialaphos resistance
BG	Bollgard
BG II	Bollgard II
Bt	*Bacillus thuringiensis*
Bta	*Bacillus thuringiensis* subspecies *aizawai*
Btk	*Bacillus thuringiensis* subspecies *kurstaki*
BXN	Bromoxynil
CaMV	Cauliflower mosaic virus
CIB	Conselho de Informações sobre Biotecnologia
CpTI	Cowpea Trypsin Inhibitor
Cry	Crystal
CSIRO	Australia's Commonwealth Scientific and Industrial Research Organisation
CTNBio	Brazilian National Biosafety Committee
DAS	Dow AgroScience
DNA	Deoxyribonucleic acid
DP	Deltapine
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EPA	United States Environmental Protection Agency
EPSPS	5-enolypyruvylshikimate-3-phosphate synthase
FAS	Foreign Agricultural Service
GMO	Genetically modified organisms
GUS	Glucuronidase

IBGE	Instituto Brasileiro de Geografia e Estatísticas
IRM	Insect resistance management
LC	Lethal concentration
MON	Monsanto
mRNA	Messenger ribonucleic acid
nos	Nopaline synthase
npt	Neomycin
pat	Phosphinothricin acetyltransferase
PIP	Plant-incorporated protectants
PPT	Phosphinothricin
RR	Resistant homozygotes
RS	Heterozygotes
SS	Susceptible homozygotes
Ti	Tumor-inducing
TP	Transgenic plants
USDA	United Stated Department of Agriculture
USDA-FAS	United States Department of Agriculture – Foreign Agriculture Service
USDA-ARS	United States Department of Agriculture – Agriculture Research Service
VIP	Vegetative insecticidal proteins

1 Introduction

Cotton is estimated to have been cultivated on more than 33.8 million hectares in over 80 countries in 2007 and 2008, yielding more than 26.2 million tons (USDA-FAS, 2007). Cropping cotton (Fig. 1) and manufacturing its products continue to be top commodities with a significant source of income for approximately one billion people despite all of the technology adopted in the field and in the factories (Heinicke and Grove, 2005; Humphrey, 2006). Hundreds of cotton varieties have been developed to permit cultivation of the crop across a broad geographic range. However, its broad distribution also exposes the cotton crop to an array of major pests that can seriously limit its production and profitability everywhere.

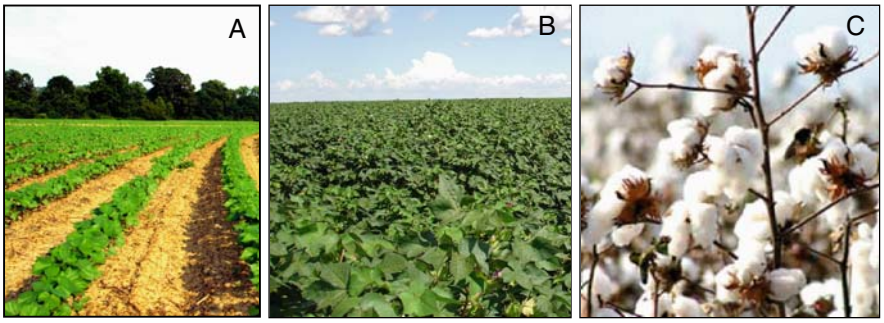

Fig. 1 Phenological stages of cotton plants: A) early season (~25 days); B) boll formation (~100 days); and C) harvest season (~160 days). Photos: J.B. Torres

Fig. 2 Lepidopteran species key pests of cotton targeted by most Bt transgenic cottons: **A**) fall armyworm (*Spodoptera frugiperda*); **B**) cotton bollworm (*Helicoverpa zea*); **C**) pink bollworm (*Pectinophora gossypiella*); **D**) cotton leafworm (*Alabama argillacea*); **E**) tobacco budworm (*Heliothis virescens*); and **F**) old-world bollworm (*Helicoverpa armigera*)

The intensive insecticide used in cotton pest management results in side effects such as human and environmental contamination, pests resistant to insecticide, and hence, lack of profitability. Thus, the economic and environmental costs of pest management in cotton continue to encourage development of alternative strategies to reduce pest attack. Lepidopteran-resistant transgenic Bt cotton has been a cost-effective strategy against the major lepidopteran species in most cases (Fig. 2), which is reflected in its rapid adoption worldwide. Because the Bt proteins toxic to insects are produced by the plants themselves, no additional equipment is required, making the technology accessible for large and small farmers. In addition, Bt cotton does not interfere with other pest control practices, making it easy to integrate with overall management strategies.

The transgenesis of plants, however, has generated a strong debate regarding the potential negative impacts on flora and fauna, especially for species related to targeted plants—wild ancestor plants, herbivores, and beneficial arthropods. Several reviews of the data have been published that evaluate possible ecological effects of transgenic crops (Risser and Mellon, 1996; Shelton et al., 2002; Obrycki et al., 2004; Lövei and Arpaia, 2005; OCallaghan et al., 2005; Hilbeck et al., 2006; Sanvido et al., 2007; Philosophical Transaction of the Royal Society of London B, Vol. 358). Although there have been some differences in the abundance of a few arthropod species in transgenic and nontransgenic cotton fields, these differences are rare and can be addressed by several possible explanations. For example, reduced parasitoid abundance in Bt crops can be due to reduction or elimination of Bt-susceptible hosts.

Fig. 3 Unsprayed experimental plots comparing transgenic Bt- and non-Bt cottons against lepidopteran attack (right front non-Bt plot lost by worm damage). Photo: J. R. Ruberson

The greater abundance of nontarget herbivore Bt crops is often related to lower pesticide inputs in Bt transgenic insect-resistant fields. The results to date suggest that genetically modified Bt cotton is safe, but that the precautionary approach is taken at each level of laboratory, field test, and commercial assessment. Marvier et al. (2007) came to the same conclusions in their analysis of the nontarget effects of transgenic commercial crops. A quick internet search for "transgenic plant and impact" and "transgenic crop and impact" through the Web of Science database resulted, respectively, in 338 and 163 publications, although this topic has only recently emerged. It shows the intense interest in this subject and the significant effort to gather scientific data for critical decision-making.

This review addresses the development and use of Bt transgenic cotton, which is designed to control lepidopteran pests (Fig. 3). The implications of this technology for cotton pest management—especially related to beneficial and nontarget pests of transgenic cotton in a multipest ecosystem such as cotton—are significant and create opportunities to improve integrated pest management in cotton. Because of several recent literature reviews, book chapters and entire books already published on the development and impact of transgenic crops, this paper will present the development of Bt-transgenic cotton and its impact on pest management for agronomists and other academic readers. It is not our objective to present extensive data and discussions of arthropod pests for specialists.

2 What Is a " Transgenic Plant"?

The terms "transgenic plants" (TP), "plant-incorporated protectants" (PIPs), or "genetically-modified" (GM) organisms are commonly encountered. Broadly, plant and animal genetically modified organisms (GMOs) are organisms that contain a gene from an unrelated organism introduced into its genome through transgenesis. In GMO plants, the transformed plant typically contains a gene from a foreign organism such as a virus, bacterium, animal, or other unrelated plant that will produce a desired character response (expression)—for instance, plants exhibiting tolerance to herbicides and resistance to insects and viruses. Genetic engineering has allowed the transformation of plants by the introduction of foreign genes, but also by increasing the expression of specific genes already present in the plant

to improve nutrition, tolerance to drought, and soil salinity, among others. Similarly, undesirable genes can be silenced, such as those responsible for flavor in soybeans (Kinney, 2003) and for secondary compounds in cotton seeds such as gossypol, which exhibits toxicity to humans (Sunilkumar et al., 2006). Identifying and understanding the mechanisms regulating genes involved in cotton plant responses to environmental stresses (Huang and Liu, 2006; Wang et al., 2007) also offer opportunities to transform varieties to reduce their vulnerability to environmental constraints such as water scarcity and soil salinity that are growing increasingly important with global climate changes.

The genetic transformation of plants has been presented as the most significant contribution to plant trait improvement since Mendel's "Experiments on plant hybridization" in 1866. And genetic engineering of crops will likely be the discovery of the 21st century that improves food production by improving yield, stress tolerance, and other plant traits such as nutrition and secondary compounds with medicinal aims.

3 Conventional and Transgenic Plant Breeding Methods in Insect Resistant Cotton

Several conventional methods such as hybridization, mutation, and multiline backcrossing have been used to improve agronomic traits in cotton, and these continue to be of primary interest (Awan 1991; Opondo and Ombakho, 1997; Carvalho, 1999; Venkateswarlu and Corta, 2001; Ahloowalia et al., 2004; Basbag and Gencer, 2007). Cultivar selection methods, such as pedigree selection, back-cross, cultivar reselection, bulk population selection, single lock descendant, and forward crossing have all been used individually or in combination (Fehr, 1987; Bowman, 2000; Bayles et al., 2005). According to Bowman (2000), nearly 45% and 100% of effort from private and public breeder programs, respectively, are centered on cultivar development in the United States, where the greatest advances have been made in transgenic cotton. Conventional plant breeding and selection methods can be time-consuming and are often not very precise (Fehr, 1987). Most agronomic varietal traits are controlled by multiple genes, whereas insecticidal traits may be controlled by single genes (Fehr 1987). Biotechnology facilitates the horizontal transfer of genetic information from one species to another, so superior traits can be directly transferred while avoiding linkage drag—the transfer of undesirable traits along with desired ones.

Conventional plant-breeding methods, which rely on natural mutation, have developed characteristics such as early maturing and heat-tolerant cotton varieties. Higher yields have been developed using hybrid vigor, multiline back-crossing and the reselection of cultivars. These characteristics are advantageous because early boll maturation allows the avoidance of damage by mid- and late-season pests such as bollworms, boll weevil, plant bug, and stinkbugs; and high-yield compensates for pest-induced losses.

Transgenic cotton varieties are grown over significant area in the countries that allow the technology, and there is a trend to increase that area by at least 16% over the 2008 season (James, 2006). Such widespread planting creates two general concerns. First, there are very few varieties of planted transgenic cotton (Table 1), thereby reducing genetic diversity—a concern even before considering the release of transgenic crops—because selection and reselection methods focus on certain established cultivars and repeated use of the same parent lines in pedigree selection, which is the most commonly used breeding method in the United States (Bowman, 2000). Limited genetic variability places extensive pressure on pest populations, and exposes the crop to a very significant risk of these resistant pests. Therefore, continued support of conventional breeding programs is needed to expand genetic diversity in transgenic crops. Second, the great demand for transgenic crops is pushing private breeders to develop transgenic varieties. Because the development of transgenic varieties already represents nearly half of the plant breeding effort, and only private companies are involved in it, this is a further erosion in the genetic diversity of cotton (Bowman, 2000). Nevertheless, despite heavy investment by private companies in the genetic engineering of cotton, much conventional breeding research still continues.

Transgenic methods can accelerate variety development, but both transgenic and conventional methods are needed to improve agronomic traits of transgenic varieties. For example, the transformed Coker variety (more details in the next section) is not a commercial variety, and back-crossing is necessary to move the resistant trait into elite commercial varieties. Conventional back-crossing was used to develop all of the initial transgenic cotton varieties of Bollgard, Roundup Ready, or Bollgard/Roundup Ready released in 1996, 1997, and 1997, respectively, and almost all subsequent varieties (Verhalen et al. 2003). Therefore, conventional breeding will continue to play a role in combining Bt protein expression and desirable agronomic characteristics (Perlak et al., 2001).

Transgenic breeding involves screening material after back-crossing or forward-crossing. Back-crossing is the most used selection method for introducing a few selected traits into elite varieties. Subsequently, forward selection improves the traits in elite varieties (Bayles et al., 2005; Adamczyk and Meredith, 2006). The resulting cultivar after back-crossing should be equivalent to the recurrent parental cultivar, except for the transferred superior trait(s). Theoretically, it is possible to recover, on average, more than 93% of the genes of the recurrent parent line after three generations, and more than 98% after five back-crossing generations (Fehr, 1987).

The best example of the link between genetic engineering and conventional breeding is the construction of stacked or pyramided cotton varieties [hereafter, "stacked" will refer to the expression of two Bt Cry genes (e.g., Cry1Ac, Cry2Ab and Cry1F) with the same insect resistance function, and "pyramided" will refer to when two genes with different functions are inserted—e.g., glyphosate tolerant gene (EPSPS) and insect resistance gene (Bollgard II/Roundup Ready® cotton)]. Bollgard® II/Roundup Ready® was generated by conventional cross-breeding of the parental lines MON88913 and MON-15985-7. The MON88913 event contains two genes encoding the enzyme 5-enolypyruvylshikimate-3-phosphate synthase

Table 1 Commercially-available lepidopteran-resistant Bt and herbicide tolerant cottons (Sources: footnote references)

Country (area covered)	Transgenic cotton	Varieties[1]	Related genes	Year of release[2]
Argentina[3]	Bollgard	NuCotn 33B	Cry1Ac	1998
(20.8%)[Bt]	Bollgard	DP50B	Cry1Ac	
(40.0%)[HT]	Roundup Ready	Guasuncho	EPSPS[4]	2002
Australia	Ingard	Sicot189i	Cry1Ac	1996
(75%)	Bollgard II	Sicot289b	Cry1Ac+Cry2Ab	2003
Brazil	Bollgard	NuOpal	Cry1Ac	
(10.8%)		Acala DP 90B	Cry1Ac	2005
China	Bollgard	NuCOTN33B	Cry1Ac	1997
(66%)		SGK321 and more than 100 similar varieties[5]	Cry1Ac+CpTI	1999
Colombia	Bollgard	NuCotn 33B	Cry1Ac	2003
(50%)	Roundup Ready		EPSPS	2004
	Bollgard/Roundup Ready		Cry1Ac+EPSPS	2007
India	Bollgard	79 commercial varieties[6]	Cry1Ac	2002
(44.48%)	Bollgard II	07 commercial varieties[6]	Cry1Ac+Cry2Ab	2005
Indonesia	Discontinued	-–		2001
Mexico	Bollgard	NuCOTN35B	Cry1Ac	1996
(61.2%)	Bollgard Solución Faena[Ht]		Cry1Ac+EPSPS	1999
	Solución Faena[Ht]		EPSPS	2005
South Africa	Bollgard	NuOpal	Cry1Ac	2002
(75%)	Roundup Ready	DeltaOpal RR	EPSPS	2003
	Bollgard II/Roundup Ready	NuOpal RR	CrylAc+Cry2Ab+EPSPS	2006
United States[7,8]	Bollgard	DP 447BG, DP 448B	Cry1Ac	1996
(92.7%)	Bollgard II	FM 960, FM 988	Cry1Ac+Cry2Ab	1997

Table 1 (continued)

Country (area covered)	Transgenic cotton	Varieties[1]	Related genes	Year of release[2]
	Roundup Ready	27 commercial varieties	EPSPS	1997
	Bollgard/Roundup Ready	19 commercial varieties	Cry1Ac+EPSPS	2002
	Bollgard II/Roundup Ready	5 commercial varieties (FM 960B2R, FM 989B2R, FM 981B2R, DP 424 BGII/RR, DP 543 BGII/RR)	Cry1Ac+Cry2Ab+EPSPS	2005
	Roundup Ready Flex	12 commercial varieties	EPSPS[8]	2005
	Bollgard II/Roundup Ready Flex	33 commercial varieties	Cry1F+Cry1Ab+EPSPS[9]	2007
	WideStrike	PHY 440, NM 1517-99 W	Cry1F+Cry1Ab	2004
	WideStrike/Roundup Ready	PHY 370 WR, PHY 470 WR, PHY 480 WR	Cry1F+Cry1Ab+EPSPS	2005
	WideStrike/Roundup Ready Flex	PHY 485 WRF, PHY 745 WRF	Cry1F+Cry1Ab+EPSPS[9]	2007

[Bt] Lepidopteran-resistant trait; [HT] Herbicide-tolerant trait.
[1]Varieties available or cultivated during last season.
[2]Refers to the first year of the trait being commercially available or planted, which means that the current variety may or not be the currently planted variety.
[3]Trigo and Cap (2006).
[4]EPSPS (5-enolpyruvylshikimate-3-phosphate synthase).
[5]Wu (Institute of Plant Protection, Chinese Academy of Agricultural Sciences).
[6]Govind Gujar (Division of Entomology, India Agricultural Research Institute, New Deli).
[7]U.S. Department of Agriculture, Agricultural Market Service – Cotton Program, Memphis, TN, August 2007.
[8]Agbios (2002).
[9]Two copies of CP4-EPSPS gene with specific promoter sequence for expression in vegetative cotton plant parts as well as reproductive parts.

(EPSPS) from the CP4 strain of *Agrobacterium tumefaciens* tolerant to glyphosate (Nida et al. 1996), while the event MON15985 is a stacked line for insect resistance. The MON15985 event was produced by transforming the Bollgard® DP50B parent variety event 531 (expressing Cry1Ac protein) using purified plasmid DNA containing the Cry2Ab2 gene from the soil bacterium *Bacillus thuringiensis* subsp. *kurstaki*. In this case, the final step required conventional breeding to join together the desired characteristics produced by transgenesis (insect resistance and herbicide tolerance) of the two parental lines. Therefore, conventional and transgenic methods are not exclusive and can be used complementarily to reduce the time needed to produce new cultivars or varieties.

4 How Transgenic Cottons Were Developed

Cotton was the first genetically modified crop to be widely cultivated, and acreage is still growing each year. Commercially available genetically modified cottons are glyphosate-tolerant [Roundup Ready®, Roundup Ready Flex®], bromoxynil-tolerant (BXN®), and glufosinate ammonium-tolerant varieties (LibertyLink®); and Bollgard® cotton (BG), stacked genes Bollgard II and WideStrike®, or pyramided varieties containing the both genes BG (Roundup Ready®) and CpTI (Cowpea Trypsin Inhibitor) insect-resistant varieties (broadly used in China) carrying the CpTI gene stacked with Cry genes conferring lepidopteran resistance. Other varieties expressing insect resistance (VipCot®) and herbicide tolerance (2,4-D) are nearing commercial release.

The development of Bt cotton started in 1981 with the development of the biotechnology techniques for plant transformation and the characterization of *B. thuringiensis* (Bt) proteins. Bt protein characterization and the cloning of Bt genes in *Escherichia coli* indicated that the expressed protein from Bt retained insecticidal activity. The high activity obtained against insects suggested that high protein expression in transformed plants might not be necessary to obtain high efficacy (Purcell et al., 2004). The next step was to transform and regenerate the cotton plant. Two methods were used to obtain the current Bt cotton varieties that express genes encoding δ-endotoxin or exotoxin (*vip*) from Bt. First, the transformation that resulted in the commercial varieties Bollgard® (Americas) and Ingard® (Australia) was made using the *Agrobacterium tumefaciens* (Firoozabady et al., 1987). Second, Bollgard® II was developed using direct DNA transfer—a method known as "microprojectile bombardment plant transformation"—or the biolistic method (McCabe and Martinell, 1993) (Fig. 4). The *A. tumefaciens* mediation method was also used to produce WideStrike® and VipCot®.

The use of *Agrobacterium* relied on the ability of this bacterium to transfer a piece of Ti-DNA into the genome of host plants (Ti stands for tumor-inducing). The genes inserted into the Ti-DNA region through Ti plasmids are cotransferred and integrated into the host genome. *A. tumefaciens* is a bacterium that occurs naturally in wounded tissues of dicotyledonous plants, causing irregular tissue growth called crown gall tumors. As a result of a crown gall tumor, the plant produces

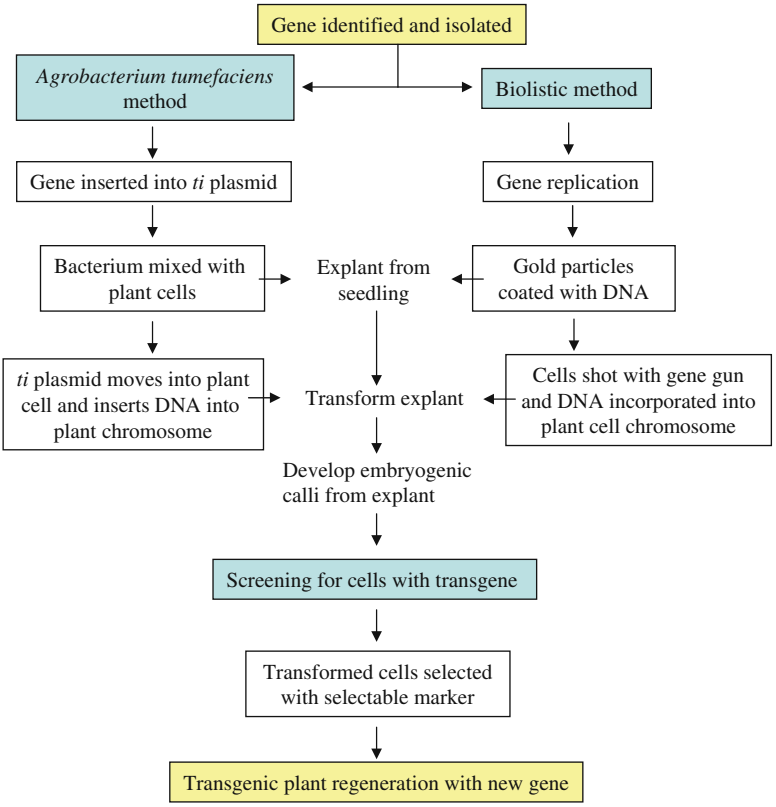
Gene identified and isolated
Agrobacterium tumefaciens method
Biolistic method
Gene inserted into ti plasmid
Gene replication
Bacterium mixed with plant cells
Explant from seedling
Gold particles coated with DNA
ti plasmid moves into plant cell and inserts DNA into plant chromosome
Transform explant
Cells shot with gene gun and DNA incorporated into plant cell chromosome
Develop embryogenic calli from explant
Screening for cells with transgene
Transformed cells selected with selectable marker
Transgenic plant regeneration with new gene

Fig. 4 Simplified steps of transgenic cotton development through *Agrobacterium tumefaciens* (e.g., Bollgard®, WideStrike®, VipCot®, and Roundup Ready®) and microprojectile bombardment plant transformation, or the biolistic method (e.g., Bollgard® II)

opines—a metabolite used by the bacterium as its food source in the medium (Tempé and Petit, 1982). Thus, by removing the genes responsible for tumor induction and opine production from the Ti plasmids, and replacing them with genes of interest, *A. tumefaciens* can vector the desired genes into plant tissues. In contrast, in biolistic transformation of a fragment of the target gene is used instead of a whole plasmid, and the target gene is coupled with a selectable marker (e.g., herbicide or antibiotic tolerance) to facilitate recognition and selection of transformed plants. The transforming genetic material is coated onto gold particles, which are then accelerated into embryonic plant tissue. Once a particle lodges in the nucleus and the introduced DNA recombines into the plant genome, the transformation is complete.

4.1 Bollgard® Cottons

The current commercial varieties of Bollgard® were produced by *Agrobacterium*-mediated transformation involving the transformation event 531 of cotton

Gossypium hirsutum L. cv Coker C312 with the vector plasmid PV-GHBKO4 (Perlak et al., 1990; Zambriski, 1992) (Table 1; Monsanto Ag. Co., St. Louis, MO, United States). Due to the difficulties in cotton regeneration from undifferentiated callus tissue, most genetic transformation of cotton is restricted to Coker 312 or related varieties. Because the Coker line is not cultivated, the resistance character must subsequently be transferred to higher-yielding and better-adapted elite varieties through back-crossing (Perlak et al., 2001). The plasmid vector PV-GHBKO4 contains two plant gene-expression cassettes. One cassette caries the Cry1Ac Bt insecticidal protein, and the second cassette carries the selectable marker (*nptII*) under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase gene (*nos*) (more details in Agbios, 2002). To overcome the disease-inducing ability of *A. tumefaciens*, this plasmid vector also contains bacterial antibiotic-resistant genes (*nptII* and *aad*). The *nptII* gene confers resistance to kanamycin and neomycin, and the *aad* gene confers resistance to streptomycin and spectinomycin. The two antibiotic-resistant genes are linked to the bacterial promoter and do not have regulatory plant sequences. They are therefore not functional and the protein is not expressed in transformed cotton plants.

Initially, transformation of cotton using the native Bt coding gene sequence resulted in lower levels of protein expression than would have been expected (Vaeck et al., 1987). Northern blot analysis revealed that the crystal protein gene mRNA species were too short to encode toxin proteins, possibly accounting for the low expression. A chimeric transgene in plants indicated that the coding region of crystal protein genes inhibits efficient expression in plants. Cry protein genes are rich in A+T, while typical plant genes have a high G+C content. Thus, a resynthesized gene eliminates potential polyadenylation signal sequences and ATTTA sequences in A-T-rich regions, and replaces the bacterial codons with plant preferred codons. These gene modifications increased expression 1,000-fold (Perlak et al., 1991; Purcell et al., 2004). Also, the optimal expression of Bt protein Cry1Ac in Bollgard was obtained by modification of the Cry1Ac1 gene. The full-length Bt protein expressed in Bollgard cotton is a hybrid molecule composed of amino acids 1–466 of the Cry1Ab protein [from *B. thuringiensis* subspecies *kurstaki* (*Btk*) strain HD-1] (Fischhoff et al., 1987), and the remainder contains amino acids 467–1178 of the Cry1Ac protein (from *Btk* HD-73) (Adang et al., 1985). Therefore, the Cry1Ac protein expressed in Bollgard differs slightly from native Cry1Ac (Freese, 2001). This Cry1Ac1 modified gene is under the control of the CaMV promoter 35S with a duplicated enhancer region (Kay et al., 1987) and the nontranslated region of the soybean alpha subunit of the beta-conglycin gene that provides the mRNA polyadenylation signals characterized as the 7S terminator sequence (Schuler et al., 1982).

The second generation Bollgard® (Bollgard® II) was transformed to contain two Cry genes that provide lepidopteran control and facilitate improved resistance management (see below). Using the biolistic method, the second Bt insecticidal gene Cry2Ab2 (Dankocsik et al., 1990), encoding the Cry2Ab protein and the selected marker *uidA* (GUS) (transformation event 15895), was inserted into the Bollgard® genome (DP50B) containing the Cry1Ac gene (event 531) (McCabe and Martinell,

1993; Doherdty et al., 2000). The transformation of Bollgard® II used the plasmid vector PV-GHBK11 that consisted of the two plant gene expression cassettes (Cry1Ac and Cry2Ab), the kanamycin gene, and the origin of replication (Agbios, 2002). The first cassette contains the Cry2Ab2 gene and the second one contains the *uidA* gene encoding the β-D-glucuronidase (GUS) reporter protein used to facilitate the selection of plants producing Cry2Ab protein (Jefferson et al., 1986; Agbios, 2002). Both genes Cry2Ab2 and *uidA* are under the control of the enhanced CaMV promoter (e35S) and the nopaline synthase gene (*nos*) from *A. tumefaciens* (Perlak et al., 2001).

Stacking Cry1Ac and Cry2Ab protein expression in Bollgard II did not affect their individual expression within the plant or over the growing season (Adamczyk et al., 2001; Greenplate et al., 2003), but increased the spectrum of lepidopteran pest control. Overall, Bollgard II expresses much higher levels of Cry2Ab than Cry1Ac within plant structures (Greenplate et al., 2003). The activity of cotton near-isoline expressing single gene Cry2Ab or stacked gene (Cry1Ac+Cry2Ab) exhibited better control against *Helicoverpa zea* (Boddie), *Spodoptera frugiperda* (J. E. Smith), *Spodoptera exigua* (Hübner) and *Pseudoplusia includens* (Walker) (Adamczyk et al., 2001; Greenplate et al., 2003). The improvement is attributed to the wider activity spectrum of Cry2Ab and to the higher levels of Cry2Ab expression in Bollgard II (Perlak et al., 2001; Greenplate et al., 2003). Similar to Cry1Ac protein, Cry2Ab is expressed throughout the plant's structures, but at negligible levels in nectar and pollen (Agbios, 2002). Hence, *H. zea* larvae fed for 72 h on squares and flower anthers experienced lower mortality compared to those that fed on bracts, petals, and squares in Bollgard but with some extended effect on squares and flower anthers in Bollgard® II (Gore et al., 2001).

4.2 WideStrike® Cottons

WideStrike® cotton originated from two events—281-24-236 and 3006-210-23—transformed for Cry1F and Cry1Ac protein expression from Bt, respectively, both by Dow AgroScience (DAS-21Ø23-5 x DAS-24236-5; Dow AgroScience LLC, Indianapolis, IN, United States). The event consisted of the Germain`s Acala GC510 cotton transformed through a disarmed *A. tumefaciens.* The WideStrike™ cotton varieties contain a Cry1Fa2-modified synthetic gene optimized to express Cry1F protein from *B. thuringiensis* subspecies *aizawai* (*Bta*), strain PS811, and the modified Cry1Ac1 gene from *Btk* HD-73, encoding the Cry1Ac protein. Therefore, the WideStrike® cotton varieties express-stacked Cry1F from *Bta* and Cry1Ac from *Btk* proteins and the genetic materials necessary for their production (EPA, 2005). The event transformation 281-24-236 (Cry1F) contains the full-length Cry1F under the control of four copies of the mannopine synthase promoter pTi5955 [(4OCS) deltamass2`) from pTiAch5 *A. tumefaciens*] and one intact copy of the synthetic plant-optimized glufosinate-ammonium resistant gene phosphinothricin acetyltransferase (*pat*) under the control of the promoter *Zea mays* ubiquitin (*UbiAm1*) used as a selectable marker inserted into the plants using the plasmid vector pAGM281. This

plasmid is a bidirectional terminator on DNA borders A and B regulated by ORF25 polyA from *A. tumefaciens* pTi15955 strain LBA 4404 (Barker et al., 1983) carrying the transgenes for insertion into plant genomes between T-DNA borders and bacterial antibiotic resistance marker (*pat*) to facilitate cloning and maintenance of the plasmid in bacterial hosts. The 3006-210-23 event (Cry1Ac) resulted from an insertion of the plasmid vector pMYC3006. This plasmid construction is almost identical to the plasmid pAGM281, except for the modified Cry1Ac1 gene and exchanged positions between the promoters *UbiAm1* and (4OCS) delta-mass2 (Anonymous, 2007). These two events—cotton 281 and cotton 3006—were back-crossed three times with the widely adapted U.S. variety PSC355, followed by one self-pollination generation each. The two back-crossed events BC3F1 were then intercrossed and self-pollinated to produce the cotton variety 281-24-234/3006-210-23, which contains genes for the expression of Cry1F, Cry1Ac, and *pat* proteins. The stacked WideStrike® cottons exhibit an increased spectrum of activity against targeted lepidopteran pests (Adamczyk and Gore, 2004).

4.3 VipCot® Cottons

Besides the δ-endotoxin crystals (Cry), *B. thuringiensis* also produces proteins with insecticidal activity during its vegetative growth, which are referred to as vegetative insecticidal proteins (vip). The vip proteins are considered the second type of insecticidal Bt proteins, and they do not form the protein crystals characteristic of Cry proteins. They are found in the supernatant fluid of vegetative Bt cultures rather than during sporulation (Estruch et al., 1996), and differ in structure, function, and biochemistry from Bt δ-endotoxins (Warren, 1997). Vip was first reported in 1996 (Estruch et al., 1996) and the studies and discovery of related genes has increased in a similar way to that of Cry proteins. To date, 76 genes coding *vip* proteins have been described (Crickmore et al., 2007). Nearly 15% of *B. thuringiensis* strains produce homologous vip3A genes encoding an 88–89 kDa protein (Estruch et al., 1996; Yu et al., 1997).

The transformed event COT102 from Syngenta Seeds, Inc. (Syngenta, Research Triangle Park, NC, United States) (SYN-IR1Ø2-7) expressing vip3A protein also relied on *A. tumefaciens* mediation in the Coker 312 cotton. The transformation consisted of introduction of the vector plasmid pCOT1 containing synthetic gene vip3A(a) from *B. thuringiensis* strain AB88 coding for the vip3A, and the gene *aph4* for hygromycin-B phosphotransferase from *E. coli*, to confer resistance to the antibiotic hygromycin to facilitate the identification of transformed lines. To optimize expression in plants, the vip3A(a) gene was modified to encode for glutamine at position 284 instead of lysine. The vector sequence also included the *aadA* (streptomycin adenyltransferase) gene, which confers resistance to streptomycin and spectinomycin, and was used as a bacterial selectable marker. The sequence of vip3A(a) gene transcription was regulated with the *actin-2* gene promoter from *Arabidopsis thaliana*, including the first exon and intron from the nontranslated

leader sequence, followed by the *A. tumefaciens* nopaline synthase (*nos 3*') terminator. The *Arabidopsis* ubiquitin-3 promoter regulating the expression of the *E. coli aph4* gene was placed in the sequence followed by the *nos 3*'synthase terminator. Then, successfully transformed cells were selected using a culture medium containing hygromycin. The vip3A protein expressed in transformed event COT102 is identical to that of the bacterial protein. Details on pCOT1 plasmid construction can be found in Artim (2003). Studies conducted with trypsin and lepidopteran gut proteases demonstrated that the 60 kDa toxic core is cleaved from the full-length 89 kDa vip3A protein, similar to the native bacterial protein (Agbios, 2002).

The VipCot® cotton lines Cot102 and Cot202 originated from back-crossing of the transformed Coker event expressing the vip3A protein that Syngenta is planning to introduce to global agriculture soon. The vip3A protein is produced in all plant tissues of the transformed events Cot102 and Cot202, and levels of protein expression do not decline with plant age, as is the case for Cry proteins (Llewellyn et al., 2007), which allows increased late-season *Helicoverpa armigera* (Hűbner) survival in Bt cotton plants expressing Cry1Ac (Olsen et al., 2005). Therefore, high levels of *H. armigera* control are maintained late into the season in VipCot®.

The vip3A proteins bind to specific receptors in the mid-gut epithelium of susceptible lepidopteran species. After binding, cation-specific pores form that disrupt ion flow, causing paralysis and death (Yu et al., 1997). The action of vip3A protein corresponds to specific binding sites in the insect mid-gut that are different from those binding sites used by Cry1Ab and Cry1Ac, eliminating the risk of cross-resistance between Cry1A and Vip3A (Lee et al., 2003; Fang et al., 2007). In addition, VipCot® has an increased target spectrum against fall armyworm, beet armyworm, cotton bollworm, old-world bollworm, and soybean looper (Fang et al., 2007). Therefore, VipCot® is considered an alternative to Bollgard® and WideStrike® cottons.

4.4 Herbicide Tolerant Cottons

Herbicide tolerant cottons are not directly associated with arthropod insect control in cotton but offer the growers the opportunity to address two of the most important problems in growing cotton—insects and weed competition—simultaneously, with minimal risk, to yield reduction. In addition to enhancing pest management in cotton farming, herbicide-tolerant cottons have made an important contribution to soil conservation practices, such as reduced tillage that benefits soil and water conservation.

There are four herbicide-tolerant cottons on the market today—Roundup Ready® and Roundup Ready Flex®, which tolerate the herbicide glyphosate; BXN® varieties tolerant to bromoxynil; and LibertyLink® varieties tolerant to glufosinate ammonium. Other varieties are under development, such as 2,4-D-tolerant varieties aimed at minimizing drift effects and offering another option for controlling dicotyledoneous weeds.

The Roundup Ready® cottons are tolerant to glyphosate [*N*-(phosphornomethyl/glycine]—a nonselective herbicide widely used for weed control, but only in the plant's early developmental stages. Thus, glyphosate can be applied as a topical application in Roundup Ready® cotton from emergence up to the four-leaf stage. After this stage, glyphosate can only be used, if carefully applied, to avoid cotton foliage and reproductive structures. Misapplication can result in fruit abortion and yield reduction due to morphological changes in reproductive structures and production of nonviable pollen (Jones and Snipes, 1999; Pline et al., 2002; Viator et al., 2003). Because of the limitations of Roundup Ready®, a new event transformation was developed that extended glyphosate tolerance through reproductive growth. This new event is referred to as Roundup Ready Flex® cotton (transformation event MON88913), or Roundup Ready® Flex. Roundup Ready® Flex varieties allow topical application of glyphosate all season long up to near harvesting time (Anonymous, 2006).

The current Roundup Ready® cotton varieties were obtained from the cotton lines 1445 (GTCot 1445) and 2698 (GTCot 1698) that were made tolerant to glyphosate after the *A. tumefaciens*-mediated transformation of the Coker 312 variety to contain the EPSPS gene. The EPSPS gene encodes the 5-enolpyruvylshikimate-3-phosphate synthase enzyme from *A. tumefaciens* strain CP4. All details on Roundup Ready® cotton transformation and screening of cotton lines 1445 and 2698 are found in Nida et al. (1996) and summarized in Agbios (2002). The first release of Roundup Ready® cotton consisted of four varieties obtained from recurrent parental back-cross in 1997.

Studies of application systems in field tests demonstrate that planting Roundup Ready® cotton results in fewer herbicide applications than conventional weed control programs, and with equivalent net revenue (Culpepper and York 1999). Additionally the RRF allows multiple judicious glyphosate applications beyond the four-leaf stage with effective weed control (Main et al., 2007). The first year Roundup Ready® Flex cotton was on the market (2006), it was planted on an estimated 800,000 hectares, predominantly in the United States, followed by small areas in Australia and China (James, 2006).

LibertyLink® cotton is tolerant to glufosinate (herbicide bialophos®) and was developed by Bayer CropScience (Bayer CropScience, Triangle Research Park, NC, USA). The LLCotton25 event was produced by *Agrobacterium* mediation using the Coker 312 cotton. The transgene was developed based on the glufosinate detoxification pathway found in the *Streptomyces hygroscopicus* fungus. This pathway is mediated by the *bar* (bialaphos resistance) gene, which encodes the phosphinothricin acetyl transferase (*pat*) enzyme that converts the herbicidal molecule to a nontoxic acetylated form (Thompson et al., 1987). The LLCotton25 event expresses PAT protein encoded by the *bar* (bialaphos resistance) gene, conferring tolerance to the glufosinate ammonium active ingredient L-phosphinothricin. The PAT enzyme in transformed plants acetylates L-phosphinothricin into a nonphytotoxic metabolite (N-acetyl-L-glufosinate). Since its discovery, the bar/pat gene system has been used to engineer glufosinate tolerance in many crops, including cotton (Keller et al., 1997).

Glufosinate ammonium is a postemergence, broad-spectrum contact herbicide and plant desiccant. Glufosinate is converted by plants into the phosphinothricin (PPT) phytotoxin. The herbicide acts by inhibiting the essential ammonia assimilation enzyme, glutamine synthetase (GS). Thus, the development of LibertyLink® has allowed us the use of glufosinate ammonium as an alternative herbicide for weed control in cotton. This additional herbicide class may help reduce the incidence of herbicide resistant biotypes.

In 1997, the herbicide-tolerant BXN® cotton developed by Calgene (Calgene Inc., Davis, CA, USA) (Agbios, 2002) was approved for use in the United States. The BXN® cotton varieties are tolerant to the herbicide bromoxynil. The oxynil family of herbicides is active against dicotyledoneous plants by blocking electron flow during photosynthesis. The BXN® cotton line was developed by *Agrobacterium* mediation with a transfer-DNA (Ti-DNA) containing a gene encoding the nitrilase enzyme from the soil bacterium *Klebsiella pneumoniae* subsp. *ozaenae*. The nitrilase enzyme breaks down bromoxynil into DBHA (3,5 dibromo-4-hydroxybenzoid acid), which does not have herbicide activity. The BXN 47 and BXN 49B varieties are available commercially.

Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO Plant Industry, Canberra, Australia) has submitted cotton lines to field test that are genetically modified to express a 2,4-D (2,4-dichlorophenoxy acetic acid) detoxification gene, which has significant potential for commercialization. The 2,4-D-tolerant cotton line was produced using *A. tumefaciens* transformation containing the plasmid pJP4 from *Ralstonia* (=*Alcaligenes*) *eutrophus*, modified to carry the neomycin phosphotransferase II (*npt II*) and 2,4-D monoxygenase (*tfd A*) genes. The *tfdA* gene was shown to encode a 2,4-D dioxygenase, which degrades 2,4-D into the inactive compounds glyoxylate and 2,4-dichlorophenol (2,4-DCP) (Streber et al., 1987) through two complex 2,4-DCP malonyl and sulfate glucoside pathways (Laurent et al., 2000). The cotton plants obtained were tolerant to three times the recommended field concentrations for the control of broadleaf weeds in wheat, corn, sorghum, and pastures (Bayley et al., 1992; Charles et al., 2007). Cotton varieties with tolerance to 2,4-D conferred by the *tfdA* gene have been developed and field-tested in Australia (Llewellyn and Last, 1996). The 2,4-D herbicide is a selective herbicide to grasses and produces strong vapor drift, causing phytotoxicity to cotton when applied nearby. Thus, 2,4-D-tolerant cotton, besides minimizing toxicity from drift, offers another option for directly applying herbicides to control dicotyledoneous weeds.

5 Potential Nontarget Effects of Bt Cottons

No other agricultural technology has been subjected to such thorough risk assessment studies before deployment as Bt crops, with hundreds of manuscripts and dozens of book chapters discussing the potential for nontarget impacts by Bt cotton. This precautionary approach, though, is fully justified. As the word "potential"

suggests, we are analyzing something that shows some likelihood of happening, and therefore is a perceived hazard with an uncertain risk. This means we need to analyze the risk vigorously on a case-by-case basis, as has been the standard practice since the beginning of Bt cotton development. In traditional risk assessment, the risk of a putative agent is defined as its hazard (its potential to do harm) multiplied by its exposure to the organism(s) of interest. With widespread planting and expression of Bt proteins throughout most of the plant, there is considerable potential for Bt to be exposed to numerous organisms of interest. However, the extent to which the organisms in question are actually exposed to the proteins will vary considerably with the ecology of the species so that toxicity of the Bt proteins to various organisms also must be considered on a case-by-case basis.

The three major categories of the potential impact of Bt cotton on the environment are: (1) weediness, (2) gene flow/out-crossing, and (3) impact on nontarget organisms (e.g., nontarget herbivores, natural enemies, pollinators, soil microorganisms, and vertebrates). The potential negative impact on the populations of natural enemies is of considerable importance to an IPM approach and corresponds to the majority of the studies on nontarget effects of Bt cotton and other genetically modified plants.

Research results have been published covering tritrophic interactions between Bt cotton, herbivores, and natural enemies (Fig. 5). In most of these studies, researchers compared Bt and conventional cotton under an insecticide regime, arguing that this approach is more realistic because it reflects the way insect communities are exposed to Bt technology in the field. Under these conditions, all studies have shown that the effect of the insecticides overrides any effect of Bt cotton.

For example, species abundance and dynamics across three seasons were evaluated for 21 foliage-dwelling (Torres and Ruberson, 2005a), and 65 ground-dwelling (Torres and Ruberson, 2007a) arthropods of importance in cotton pest management. The results showed no differences in the ground-dwelling arthropod communities between cotton types, and one shift in abundance of one foliage-dwelling predator—the convergent ladybird *Hippodamia convergens* Guerin-Meneville—between cotton genotypes. Significantly larger populations of convergent ladybirds were found in non-Bt cotton (Torres and Ruberson, 2005a). The mechanism behind the population shift was investigated and the results indicated no reduction of ladybird population based on Bt cotton. After ruling out other possible effects (such as prey availability and movement between fields), a toxicity test of field-collected adult ladybirds *Harmonia axyridis* (Pallas) and *H. convergens* showed that use of the insecticide λ-cyhalothrin in non-Bt cotton to control bollworms was the cause of the shift. The lowest recommended dose of λ-cyhalothrin killed 100% of *H. axyridis*, while no mortality of *H. convergens* was observed even at the highest recommended dose. These results suggest that the change was caused by competition relief. Using the insecticide early in the season reduced the superior competitor, *H. axyridis*, allowing immature specimens of *H. convergens* to flourish, while in untreated Bt cotton fields the competition suppressed *H. convergens* (Torres and Ruberson, 2005b). Insecticide use in non-Bt cotton is often used to explain greater populations in Bt cotton, but rarely the other way around.

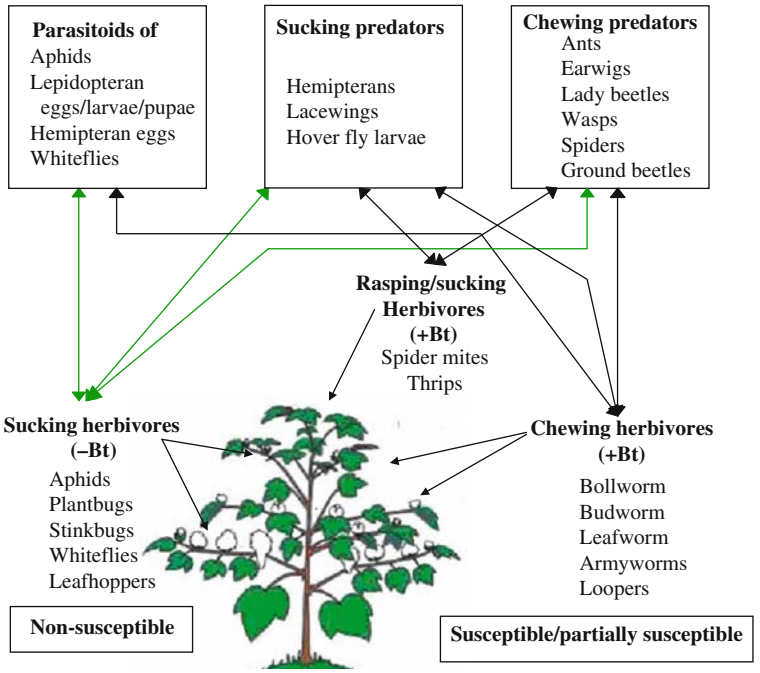

Fig. 5 Simplified food web in cotton ecosystem showing potential direct protein exposure to the third trophic level (+Bt) and host/prey quality potential impact (–Bt). The boxes representing the third trophic specify only major natural enemy groups found across growing cotton regions that comprise predators and parasitic species. Dark arrows stand for prey conveying Cry protein from Bt cotton plants (+Bt); and green arrows stand for prey not conveying protein from Bt cotton plants (–Bt)

Field experiments testing the effects of Bt cotton cannot distinguish between population-level, prey-mediated, or direct Bt protein effects. Despite all these possible modes of action, the results have varied from an increase in specific predator taxa (to no effect) to a decrease in specific predator taxa in relation to Bt cotton. The lack of consistency may be due to a variety of confounding sources apart from the disruptive effect of insecticides, including small plots, the number of seasons surveyed, the number of samples taken during the crop season, and specific agronomic practices. The statistical power of many studies is also in question, as many studies can only detect differences in abundant species. A small change, or changes in rare taxa, is much more difficult to detect.

While transgenic cotton leaves an overwhelmingly smaller ecological footprint than any insecticide-treated cotton, it is still important to know if and how Bt cotton effects the community, even it is only a slight effect. Since Torres (2005), 13 papers have been published on the effect of Bt cotton on insect communities. Of these, only four exclude the influence of insecticides (Naranjo, 2005a, b—U.S. cotton; Whitehouse et al., 2005—Australian cotton; Cui et al., 2006—Chinese cotton). Cui et al.

(2006) looked at the effect of Bt cotton on the stability of the invertebrate communities and their diversity in Chinese cotton. They concluded that, if anything, Bt cotton is more stable. Whitehouse et al. (2005) reported on invertebrate communities in Australian cotton at four sites spread over three seasons. They found a 4.5% change in the composition of species in unsprayed conventional and Bt cotton. This figure included the strong reduction in *Helicoverpa* found in Bt crops, and may largely reflect the response by the invertebrate community to this loss (Whitehouse et al., 2005). When the abundance of key species was compared across all sites, four species groups (apart from *Helicoverpa*) showed a small but consistent drop in abundance in Bt crops. Two of these were flies (fruit flies [Drosophilidae] and Frit flies [Chloropidae]), and two were Hemiptera (Jassids [Cicadellidae] and damsel bugs [Nabidae *Nabis kinbergii* Reuter]), none of which are directly effected by the Bt Cry proteins used in these experiments. Damsel bugs are generalist predators in cotton, so their numbers could have fallen because of the reduction in *Helicoverpa*, which is one of their prey. The role that frit flies play in cotton is unknown (some species are a pest in wheat, whereas others are benign) so the reason for the drop in their abundance, and its consequences (if any), are unknown. Naranjo (2005a) working with U.S. cotton, also reported a slight drop in the abundance of *Nabis alternatus* Parshley in Bt cotton over the seasons from 1999 to 2003, and a slight drop in the ladybird *Hippodamia convergens* in Bt cotton. In a companion study, Naranjo (2005b) reported that despite these changes, there was no difference in the ecological functions of the Bt and conventional arthropod communities.

Whitehouse et al. (2007) working with unsprayed plots of vip and conventional cotton again found that the transgenic crop only accounted for a small proportion of the variance (2–7%) of the arthropod communities in conventional and vip plots. Dively (2005) also found a slight change between the vip and conventional community that he related to indirect plant-mediated factors, density responses to prey availability, and the absence of insect damage.

Laboratory studies on the adverse effect of Bt proteins or Bt-reared prey on natural enemies has been patchy, with some groups (e.g., braconid wasps and green lacewings) receiving a lot of attention, and others (e.g., flies) receiving none (Lövei and Arpaia, 2005). In cotton, the adverse effect of Bt proteins or Bt-reared prey has been found in only four arthropod predators in three laboratory studies. The predators (larvae of the green lacewing *Chrysoperla carnea* (Stephens); the predatory heteropterans *Geocoris punctipes* (Say) and *Orius tristicolor* White; and the ladybird *Propylea japonica* (Thunberg)) were negatively affected when fed diet containing Cry1Ab or by prey-fed Bt cotton. Later studies revealed that the effect on *C. carnea* was the result of suboptimal prey quality and not the toxicity of Bt protein (Dutton et al., 2002; Romeis et al., 2004).

Field-collected *G. punctipes* and *O. tristicolor* of an unknown age had lower survival rates when confined in the laboratory and fed beet armyworm (*S. exigua*) larvae reared on Bt cotton (Ponsard et al., 2002). In these experiments, the authors recognized problems with the caging method, especially prey size and moisture offered to the predators during the experiment. These factors can cause variation in predator survival. In a long-term field study, Torres and Ruberson (2006) evaluated

the development and reproduction of *G. punctipes* over two cotton seasons when fed young *S. exigua* larvae that were consuming Bt cotton (expressing Cry1Ac). In contrast to the findings of Ponsard et al. (2002), the life history of the predator was not affected by Bt and non-Bt cotton plants, nor by prey acquiring the protein. *Orius* species given a diet of either pollen collected from Bt plants, or prey fed on Bt plants produced no measurable negative effect (Zwahlen et al., 2000; Al-Deeb et al., 2001).

Weight gain and survival of the ladybird *P. japonica* were reduced when predators were reared on second-instar *Spodoptera litura* (F.) from Bt as opposed to non-Bt cotton (Zhang et al., 2006a). On the other hand, in this same study, the authors presented results comparing a variety expressing dual genes (Cry1Ab+Cry1Ac). When fed *S. litura* prey from this Bt cotton, *P. japonica* experienced greater weight gain (14%) and larval survival (20%) compared to ladybird larvae given prey that were fed the single-gene plant, even though the *S. litura* prey reared on the dual-gene plants contained ∼26% more protein than those reared on the single-gene plants. Therefore, the predator larvae grew and survived better on prey containing greater amounts of Bt protein to the third trophic level. This result suggests other factors besides the proteins themselves were affecting weight gain and survivorship, and contaminated prey were affecting the outcomes of these experiments during their short evaluation periods (ca. 72 h predator feeding on 24 h Bt-fed prey). Further, this same predator, *P. japonica*—when fed either the cotton aphid *Aphis gossypii* Glover reared on Bt cotton varieties, or the plant-hopper *Nilaparvata lugens* Stål, reared on Cry1Ab-transgenic rice—exhibited similar developmental, survival, and fecundity rates as predators reared on prey from non-Bt plants (Zhang et al., 2006b; Bai et al., 2006).

Lepidopteran parasitoids are often reported at a lower density in Bt crops (Baur and Boethel, 2003; Manichini and Lozzia, 2004; Dively, 2005) than conventional crops. Explanations for this difference include the host lepidopteran larvae susceptible to Bt proteins die before the parasite could complete development and low host quality delaying parasitic development. Parasitism of pests fed Bt plants or a diet containing Bt proteins, but only partially susceptible to Bt, generally allow parasitoids to successfully complete development even though they suffer sublethal effects, such as delayed larval development, and lower pupal and adult weight. This response has also been reported for parasitoids reared on hosts treated with Bt formulations, where hosts typically die before parasitoid development is complete (Blumberg et al., 1997; Atwood et al., 1999; Erb et al., 2001). These adverse effects appear to be due to reduced host suitability rather than direct effects of the Bt protein. Parasitoids developing in resistant strains of host species otherwise susceptible to Bt suffer no adverse sublethal effects (Schuler et al., 2004). Additionally, direct ingestion of protein by parasitoid adults does not affect their survival (Liu et al., 2005). Therefore, no direct adverse impact of Bt proteins produced by transgenic plants on parasitoid larvae has been found. Reduced quality of intoxicated hosts (smaller size, and possibly lower food quality) seems to be the cause of sublethal effects. Parasites specific to hosts targeted by Bt proteins, such as the bollworm complex, are at risk for population reduction due to a total failure of parasitism

(host dying before parasitic development), or sublethal effects resulting from poor host quality (Yang et al., 2001; Lu et al., 2004). However, these results are comparable to the outcome for hosts treated with insecticides (including commercial formulations of Bt; Blumberg et al. 1997) or reared on conventional resistant varieties (Baur and Boethel, 2003) that kill the host during parasitic development, or reduce host quality.

There is little evidence that Bt proteins are toxic to nontarget organisms. The Bt Cry proteins expressed in Bt cotton plants can be acquired by nontarget herbivores, but nonsusceptible herbivores lack the specific binding sites and lack the protease to activate the protoxin. In addition, the Bt protein can move through digestive tracts of at least some nontarget species, to be excreted in feces or honeydew (Bernal et al., 2002; Howald et al., 2003; Obrist et al., 2005; Torres et al., 2006). Herbivores living in cotton plants exhibit various feeding behaviors that directly affect the amount of protein they acquire from the plants (sucking herbivores obtain less Bt protein while chewing herbivores obtain more protein). In addition, Bt protein may or may not accumulate in the herbivores, influencing whether the Cry protein is conveyed to upper trophic levels (Torres et al., 2006; Zhang et al., 2006a, b; Torres and Ruberson, 2007b). Predators feeding on contaminated prey items are not affected, although indirect effects due to sick or suboptimal prey would be expected (Dutton et al., 2002). Because most predators in cotton fields exhibit polyphagous feeding behaviors, the effect of suboptimal prey can be ameliorated by consumption of prey items not targeted by Bt proteins in the diet, hence there is no net negative impact on predator population dynamics as shown in reported field surveys.

6 Resistance and Resistance Management

The season-long and systemic expression of Bt proteins in plants, and the extensive geographic adoption of Bt cotton create an environment that is favorable to the selection of Bt resistance. Additionally, lepidopteran pest species of cotton, such as *P. gossypiella* and *A. argillacea*, are nearly monophagous on cotton and are widespread over certain cotton-producing regions. These pests would be under heavy selection pressure to develop resistance if Bt cotton were exclusively planted. On the other hand, the polyphagous pests such as *H. virescens*, *H. zea*, and *Helicoverpa punctigera* (Wallengren), and *H. armigera* should be under less pressure for resistance, because there are other available non-Bt hosts upon which these moths can breed either during the Bt cotton season or between cotton seasons. However, favorable conditions for resistance still would occur if the area planted with Bt cotton was much greater than that of alternative food plants and/or if another Bt-transgenic host crop were planted in the same area (e.g., corn/maize). Of additional concern is that *H. armigera*, despite its catholic food preferences, is notorious for developing resistance to chemical insecticides. Therefore, strategies to reduce selection for resistance to Bt proteins have been adopted under two scenarios: (1) when only Bt cotton is planted, and (2) when it shares the area with other

Bt-transgenic crops such as corn or maize. Both of these scenarios come into play in the midwest region of Brazil, for instance, where Bt cotton is planted in continuous farms over 1,000 ha each, and Bt corn/maize is present as a nearby crop. In such an environment, it is a real challenge to manage the system to delay resistance.

Bt protein is expressed differently according to the plant structures. For example, expression of Bt protein is low in petals and bracts and almost negligible in pollen. Therefore, individuals in the population that occasionally feed on these parts can experience higher rates of survival (Brickle et al., 2001; Gore et al., 2001) by being exposed to sublethal doses. Protein expression in plants can also be affected by environmental factors and cropping practices such as temperature, rainfall, soil fertility and salinity, varieties, and the use of growth regulator hormones (Greenplate, 1999; Adamczyk and Sumerford, 2001; Coviella et al., 2002; Greenplate et al., 2003; Chen et al., 2005; Jiang et al., 2006; Torres et al., 2006; Pettigrew and Adamczyk, 2006; Oosterhuis and Brown, 2004). In addition, not all plants in a field of Bt cotton will express the Bt proteins. A small percentage will process none. These plants provide chances for susceptible larvae.

The lessons learned from past experience with chemical insecticides have persuaded the industry to use a precautionary approach with regard to resistance management. Both specialists and companies that developed the Bt technology are continuously vigilant for the presence of resistant individuals in field-targeted pest populations, despite the great success for Bt cotton and other crops carrying Bt genes. The companies owning the technology and regulatory agencies, therefore, are demanding that farmers planting their transformed varieties follow a strict code of practice to sustain the crop's efficacy.

The environmental advantages of using formulations of the Bt proteins over other insecticides for pest control are well-known (Glare and O'Callaghan, 2000), hence it is widely applied in agricultural, forestry, and urban ecosystems. As a result, a few species of lepidopteran larvae resistant to *B. thuringiensis*' commercial formulations have been detected. In response, strategies for delaying resistance appearing in Bt crops have been developed (Roush 1998; Gould and Tabashnik, 1998). Additionally, resistance to commercial formulations of Bt has been linked to cross-resistance of Cry proteins in *Plutella xylostella* (L.) (Tabashnik et al., 1990, 1994). This result and others fueled concerns for insect resistance management (IRM) for Bt crops.

A study using *Spodoptera exigua* from Arizona (United States) showed that field populations exhibit different levels of susceptibility to Bollgard® (Cry1Ac protein; Moulton and Dennehy, 2001). Further laboratory selection (conducted by the same authors) over three generations with three populations of *S. exigua* showed an increase in resistance to Bollgard® by 32%, 298%, and 716%, which indicates a species with a high propensity to develop resistance.

With the pest *H. armigera,* the problem is even more complicated. In the Hebei and Shandong Provinces of China, there has been a steady increase in tolerance of *H. armigera* to Cry1Ac. Models based on results from this region indicate high levels of resistance to this protein within 11–15 years if effective resistance management is not undertaken (Li et al., 2007). In addition, Gunning et al. (2005)

reported that field populations exhibiting significant variability in Cry1Ac susceptibility (silver strain) can be selected to have a resistant factor ($LC_{resistant}/LC_{susceptible}$) of 150 and 275 for LC_{50} and LC_{99}, respectively. That is, 70% of larvae were able to survive feeding on Bt cotton (Ingard® Cry1Ac). The resistance mechanism in the *H. armigera*-selected strain seems to be increased esterase sequestration of the protoxin in the gut of the larvae—a different mode of resistance from those already reported based on modifications of the receptor-binding site or alterations in the proteases that cleave the protoxin. However, after seven seasons of planting Cry1Ac Bt cotton in Australia, no field failures have been observed for *H. armigera*, and the monitoring program indicates that the frequency of genes conferring resistance to the Cry1Ac protein is very low (Mahon et al., in preparation).

Ingard®, which contains only the Cry1Ac protein, has been replaced in Australia by Bollgard® II, which also contains the Cry2Ab protein. However, the initial frequency of the allele for resistance to Cry2Ab occurred quite frequently in natural field populations (Mahon et al., 2007), suggesting that the risk of developing resistance to this gene is higher than expected. Despite this, there has been no evidence gathered from intensive monitoring programs that indicate that this frequency is increasing in field populations.

Laboratory selection of a homozygous resistant population of *H. armigera* to Cry2Ab showed that resistance was due to a single autosomal gene and that is was recessive. Homozygous individuals resistant to Cry2Ab are not affected when feeding on Cry2Ab cotton leaves, but maintain susceptibility to Cry1Ac and do not show cross resistance with the commercial Bt product Dipel®, which contains multiple Cry1 and Cry2 proteins (Mahon et al., in preparation). This and other results corroborate that Bt cottons producing dual proteins are crucial for resistance management of transgenic crops (Roush, 1998; Gould and Tabashnik, 1998; Zhao et al., 2003). However, if *H. armigera* increases its esterase response (a widespread resistance mechanism in insects) and neutralizes Cry1Ac proteins, selection pressure on the second protein, Cry2Ab, will become critical and IRM will be more difficult. We highlight this fact because the idea behind the dual-protein (stacked Bt cotton varieties) strategy is that a particular Bt protein is active at one binding site, while a different protein is active at another binding site; and the combination of the two modes of action should delay resistance when relative to a single protein. Therefore, unrelated modes of action of the two proteins are crucial for the dual protein strategy in IRM to work. When an esterase reaction neutralizes one of the proteins, the dual-binding site strategy is compromised, and protection is provided solely by the remaining protein in the dual variety, making it functionally a single-protein construct. For this reason, the susceptibility of *H. armigera* to Cry2Ab (Li et al., 2007) needs to be monitored very carefully.

Parallel to the development of Bt cotton, resistance-monitoring programs targeting major pests have been developed worldwide in Bt cotton growing regions (see special issue 95 of Journal Invertebrate Pathology 2007). Thus, in anticipation of further development of pest resistance to Bt cotton, several factors that could increase selection for resistance have been investigated and action has been taken to

mitigate their influence. Eight practices with this potential (Roush, 1998) are known as the gene deployment strategy, but due to lack of both scientific information and technological feasibility, few have been adopted. Results from modeling the gene frequency of resistance in *P. gossypiella*, *H. virescens*, and *H. armigera* suggest that to restrain the resistance of lepidopteran pests to Bt cotton, there must be: (1) a high dosage expression of Cry proteins in plants much higher than LC_{50} (concentration of protein to kill at least 50% of a population) toxic to the target pests; (2) refuge areas planted with non-Bt cotton and managed according to the recommendations (see below); and (3) dual proteins expressed with stacked genes.

A monitoring program involves collecting large numbers of individuals of key target pests from areas with heavy adoption of the Bt crop and testing their survival in a bioassay in which they are fed purified Cry protein expressed by the plant. This procedure requires that a baseline of susceptibility of the pest to Bt has been established prior to planting Bt cotton (or any other Bt crop). The baseline susceptibility data are used to track changes in the population's susceptibility to Bt through bioassays of collected insects. The baseline susceptibility of major target pests of transgenic cotton from different regions has been investigated (Jalali et al., 2004; special issue 95 of Journal Invertebrate Pathology 2007). And, for Bt-derived commercial products, the development of resistance is suspected when the difference between tested populations and the control is higher than 10 times. Differences lower than 10 times are unreliable due to variability among Bt subgroups and insect tolerance (Glare and O'Callaghan, 2000).

One of the problems when monitoring for resistance is that by the time it is detected, it is already too late. To overcome this problem, Stodola and Andow (2004) developed the F2 screening test. This is where eggs collected in the field are raised to adult moths and form mating pairs. The progeny of the pair (F1) are then raised to adulthood and form mating pairs with their siblings. The offspring of these unions (F2) are then tested for resistance. If one of the field-collected eggs carries a recessive resistant gene, then 1 in 16 of the F2 generation would be resistant.

Resistance-monitoring programs for *H. virescens*, *H. zea*, *H. armigera*, and *P. gossypiella* and Cry1Ac, Cry2Ab, and Cry1F are highly recommended and supported by biotech companies (Sivasupramanian et al., 2007). Also, all sectors involved in cotton production encourage growers to report control failures to initiate investigations of the causes. The monitoring program plays an important role in IRM because a quick remedial action plan can be adopted if resistance is identified. In addition, all sectors in cotton production need to be involved because of the challenge of monitoring the vast areas planted in Bt cotton. For example, in India and China, 44 and 66% respectively of the area planted in cotton is Bt (Table 1). That equates to over three million hectares in each country.

Structured refuges combined with high-dose strategies are the most recommended resistance management methods and are compulsory for growers cultivating Bt cotton. Several points need to be considered if a structured refuge/high-dose strategy is to be successful in delaying resistance appearance. First, one has to consider the size of the area and layout of the refuge fields, the distance between

the fields, pest biology, and neighboring crops (other Bt or non-Bt crop). In addition, the higher-dose strategy assumes that resistance to Bt is recessive and is conferred by a single locus with two alleles, resulting in three genotypes [susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR)], so that when resistant (R) survivors from Bt cotton fields cross with susceptible survivors (S) from non-Bt cotton fields or from other non-Bt crops, the offspring are susceptible. In most cases, the resistance genes in the field are partially or completely recessive (Tabashnik et al., 2000; Carrière et al., 2004), and this has been confirmed in laboratory-selected populations (Mahon et al., in preparation). The high-dose strategy also assumes that there will be low initial resistance allele frequency and that there will be extensive random mating between resistant and susceptible adults. Resistance allele frequencies can only increase over time if either heterozygotes are partially resistant, or there are resistant homozygotes in the population. Field-adopted refuges (non-Bt cotton) and other recommended strategies help reduce the number of resistant homozygous individuals by allowing them to mate with susceptible individuals and generate heterozygotes (Gould et al., 1997). Therefore, the strength of these heterozygotes will have a major impact on the rate of resistance evolution. If heterozygous (RS) individuals are produced and mate with susceptible individuals (S) grown in the refuge, only in the F2 generation will there be a probability of 1/16 individuals that are homozygote resistant (RR). The remaining 6/16 RS and 9/16 SS will be killed by the high dose of protein expressed in the plants. To maintain such proportions and the sustainability of the refuge as a delaying strategy for resistance, a subpanel of the United States Environmental Protection Agency recommends a ratio of 1 RR moth per:500 SS adult moths in an area (EPA, 2000). Therefore, refuge success relies on the assumption that a high dose kills all heterozygotes, signficantly delaying the development of a resistant population. If over 90% of heterozygotes are killed, 10% of the population are susceptible (developed in non-Bt cotton refuges—c.a., a refuge of 10%) and resistant alleles in the population are initially 10^{-3}, then Roush (1998) estimated that resistant individuals ($>$50% resistant) would occur after 40 generations or over 100 generations in areas adopting 50% refuge. However, the author noted that in species exhibiting potentially lower heterozygote mortality, such as the *Helicoverpa* species, resistance may be attained in about 20 generations when single-protein varieties are adopted. Therefore, maintaining the individuals carrying genes for susceptibility in the population is the major goal of the structured refuge/high-dose strategy.

Despite the acceptance of the predictions derived from models, biological and environmental variables always interact, and may modify the final results. Because of this and other uncertainties, the precautionary approach has been the main driving force of IRM since its adoption. If the dose of the protein is not efficacious, or the expression of the protein is quite variable, then partially resistant (e.g., heterozygous) individuals could survive, thus increasing the frequency of resistant alleles in the target-pest population. For this reason, the multiple IRM strategy is important.

A low dose of the protein presents some risks relative to a high dose (Roush, 1998; Gould and Tabashnik, 1998). A high dose assumes that plants express at least

25 times more protein than necessary to kill a susceptible target larva, but it has also been suggested that 50 times more is ideal (Caprio et al., 2000). This is cause for concern because most planted varieties of cotton worldwide (Table 1) express a single protein (Cry1Ac). Although Bt plants produce a high dose of this protein for *H. virescens*, *H. armigera*, *P. gossypiella*, and *A. argillacea*, the dosage is not high for *H. zea* and some other lepidopteran cotton pests. Therefore, dual protein expression is considered a more viable strategy than single protein expression in delaying the evolution of insect resistance, as the proteins expressed in Bt cotton plants must meet a certain level of expression. Most importantly, they should be as distantly related as possible, especially with respect to the mode of action, to discourage cross resistance.

Gene-stacking with Bt Cry and Cowpea Trypsin Inhibitor (CpTI) genes has been widely adopted in Chinese cotton varieties (Table 1). In other regions, dual protein use, such as Bollgard® II (c.a. Cry1Ac+Cry2Ab) and WideStrike® (Cry1Ac+Cry1F) varieties, is likely to increase (Table 1). The Cry2A protein in Bollgard® II was selected because it lacked immunological cross reactivity with the Cry1Ac protein already inserted in Bollgard® (Perlak et al., 2001). As indicated by exploring the Cry and CpTI combination commercially in China, other proteins with insecticidal activity can be fused into Bt plants to enhance the toxicity of the Cry proteins and to improve IRM. The combination of Cry1Ac with the galactose-binding domain of the nontoxic ricin B-chain (BtRB) furnished additional binding sites for the protein, resulting in significantly more toxicity than Cry1Ac alone (Mehlo et al., 2005). This fusion also broadened protein activity against insects that are not usually susceptible to Bt. In addition, VipCot® (expressing vegetative insecticidal proteins with a different mode of action) is soon coming to the market. Dual proteins, which impose different modes of action on the target pest, have the potential to significantly delay resistance in pests, even with the use of smaller refuge areas (Roush, 1998).

The basic recommendations of the refuge are very similar for all regions planting Bt cotton, but with some variation with respect to both planting more than one transgenic Bt-crop, and specific recommendations from committees responsible for allowing Bt-crop cultivation in each country. For instance, despite all the options available, the Brazilian National Biosafety Committee (CTNBio) determined that only the 20:80% refuge strategy (see details bellow) for Bollgard® I was feasible in Brazil in light of current knowledge.

Refuge strategies have been based on U.S. and Australian Bt cotton production systems where Bt cotton was first cultivated (EPA, 2000). Therefore, all other countries planting Bt cotton derived their recommendations from studies and models of U.S. production systems. The structured refuges consist of a portion of the field seeded with non-Bt crop. Briefly, the 05:95% refuge consists of planting 5% of the area with non-Bt cotton in a band width of at least 45 m (ca. 150 feet). This non-Bt area must not be treated with sterile insects, pheromone, or any insecticidal spray for lepidopteran larvae targeted by Bt cotton. Practices to control other pests should be adopted, but any insecticide used must not be effective against the Bt cotton target pest. Preferably, the same cotton variety should be used to match phenology, such

as the timing of flowering, maturing date, etc. The crops should also receive similar agronomic treatment such as fertilization, irrigation, weed control, and termination practices. In addition, the refuge area should be close to the Bt crop, and must be no further than 800 m (½ mile) away or separated from the Bt field by physical barriers such as large rivers, natural vegetation, or planted forests, etc.

The 20:80% refuge requires that 20% of the area be cultivated with a non-Bt variety. Almost all practices and observations recommended to 05:95 are valid for 20:80 refuges with some exceptions. The 20% non-Bt cotton area may be treated if needed with insecticides, excluding Bt products and pheromones for Bt cotton target species. The 20% refuge area must be no more than 1,609 m (1 mile) from Bt fields, and preferably closer than 800 m (½ mile).

The 05:95% embedded refuge consists of planting 5% of the area with non-Bt cotton within a field of Bt cotton and not at the edge or at some distance from the field. All the recommendations regarding varieties and agronomic practices and land area for 05:95% not embedded and 20:80% refuges should be considered. The in-field embedded refuge is only recommended for pink bollworm, and therefore has only been adopted where this is the major pest targeted by Bt cotton, such as Arizona in the United States (Carrière et al., 2004). The in-field refuge consists of planting at least one row of non-Bt cotton for every six to ten rows of Bt cotton. The seeding process can be made by setting one row of the planter with Bt cotton seeds and the remaining with non-Bt cotton. The embedded rows are cultivated using all the same agronomic practices for non-Bt cotton, including insecticide sprays, except Bt products.

Planting other Bt crops adjacent to Bt cotton requires changes in refuge deployment, particularly when the target pests use both plants as hosts. For instance, if Bt maize is planted in cotton growing areas, a non-Bt maize refuge of 50% of the area is recommended to delay resistance of bollworm and corn borer (Hardee et al., 2001; IRM Guide, 2004), as opposed to a refuge of 20% recommended for areas not planting Bt cotton.

The IRM program has to be within the IPM context, which also manages volunteer Bt plants developing outside the growing season. This is particularly the case in tropical regions where old plants and seedlings are not killed by freezing conditions and can still grow between seasons or in the following season as weeds in other crop fields—and host pests. Thus, cultural management of the crop is very important. Several recommendations have been made addressing the management of the crop to enhance IRM: (a) use a nonselective herbicide other than glyphosate when planting herbicide-tolerant varieties prior to seeding the next crop; (b) establish a planting window when cultivating multiple Bt-crops in the same area; (c) synchronise the seeding period; and (d) make crop residue destruction mandatory to avoid continuing generations on other host crops and residues. Overall, cotton is a long-season crop, taking more than five months to mature, while other annual crops—mainly grasses—develop within three months. Therefore, cotton can be used as a sink host in the relationship with other non-Bt and Bt crops serving as sources. In addition to the cultural recommendations, it is important to consider the restrictions discussed above for refuges when applying insecticides. Finally, give preference to varieties

expressing dual proteins when available, especially because different transgenic cotton varieties have been produced with Cry1Ac, Cry2Ab, Cry1F, Cry1A+CpTI, VIP, Arrowhead PI, and pea lectin in different countries (Perlak et al., 1990; Estruch et al., 1996; Perlak et al., 2001; Wang et al., 1999; Huang et al., 2001; Cui et al., 2002).

7 Bt Cotton Perspective in Brazil

Brazil planted 120,000 hectares in the first season of commercial Bt cotton (James, 2006), and it is estimated that a half million hectares will be planted in the 2007/2008 season (CIB, 2007). The major challenge for the first season was to obtain certified seeds, since only two Bt cotton varieties were available: Acala DP90 B and NuOpal (Table 1).

In Brazil, there are four major cotton-growing regions: the Northeast, Central, Midwest, and Amazonian regions. These regions cover a wide range of different environmental conditions with different target and nontarget Bt cotton pests. Therefore the challenges and pest management strategies for Bt cotton are specific for each region.

The Northeast region of Brazil planted 353,581 hectares and accounted for 29% of cotton production in 2007 (IBGE, 2007). However, despite belonging to the Northeast region, the western part of the Bahia state, which is the second largest producer state of Brazil by planting 257,377 ha, is more similar to the Midwest region in terms of climate, cotton varieties, and the use of technology. Therefore, the northeastern climatic and cropping system is, in fact, only 76,891 hectares. The Northeast region area is characterized predominantly by family agriculture with small land holdings and the absence of machinery and heavy external inputs. The main limiting factor of widespread cotton planting in this region is the unreliable rainy season. Pest problems in the Northeast region are relatively small compared to the other Brazilian cotton regions and most production loss is caused by cotton boll weevils, aphids, cotton leafworms, and pink bollworms (Ramalho, 1994). The major challenge for the adoption of technology in this area is the level of farmer education and limited financial resources (Fontes et al., 2006). Moreover, the adoption of Bt cotton may be delayed because of the need to insert the Bt genes into varieties suited to the environmental conditions and cropping system of the region. It is unlikely that the small seed market in this region will be attractive to private seed companies. Therefore, development and adoption of transgenic cotton varieties in the Northeast region will probably depend on public Brazilian national biotechnology laboratories, such as the Embrapa Cotton Research Centre, which is the main source of seed production and marketing for this area.

The Central region, which is comprised of the states of São Paulo and Paraná, planted 66,450 hectares in 2007. The cotton production in this region is characterized by farms from 10 to 15 ha, but some can reach over 500 ha. Farmers usually adopt advanced technologies and are likely to expand areas planted with Bt cotton during the 2007/2008 season. In this region, limiting factors include

disease and pests. The key pests are boll weevils, cotton leafworms, tobacco budworms, and pink bollworms (Santos, 1999). The most commonly planted varieties are susceptible to blue disease transmitted by aphids (Cia and Fuzatto, 1999). The constant infestation of the boll weevil in this region slows down Bt cotton adoption.

The Midwest region cultivates the largest area, and has the greatest production and yield of cotton in Brazil. The farm acreage with cotton ranges from 5 ha to over 10,000 ha. In the state of Mato Grosso, the largest producer, most of the farms are larger than 1,000 ha. The region comprises the Cerrado biome, which has humid summers and dry winters. The cotton growing season covers the humid summer starting in November and ending in April, with harvest from May to August depending on the local elevation (Fontes et al., 2006). Farmers or groups of small farmers use modern technologies for all farming operations including their own ginning. The limiting factors include diseases and pests. Depending on which variety is planted, up to seven fungicide sprays are required. Due to high yield and fiber quality, most growers prefer the variety (CNPA Ita 90) that is susceptible to blue disease. Hence, aphids are considered key pests in these areas. The predominant lepidopteran pests include armyworm species (*Spodoptera* spp.), cotton leafworms, tobacco budworms, and pink bollworms (Fig. 2). The boll weevil is absent in some areas and farmers use practices designed to minimize its spread. This and the Meridian region are most likely to show the strongest adoption of Bt cotton in Brazil.

In the Amazonian region, the southern states such as Tocantins and Acre and the far southern parts of Amazonian and Pará states are the areas planting cotton. Except the states of Tocantins and Acre, the other areas plant only a small proportion of Bt cotton.

Even though Brazil is allowing Bt cotton to be planted 10 years after it was initially cultivated in other major producing countries (China, India, the United States, Australia, etc.), only the first generation of Bt cotton is currently available (Bollgard expressing Cry1Ac).

Another feature of Bt cotton in Brazil was the care taken to identify areas considered centers of native or naturalized cotton species. Transgenic cotton has been prohibited from those areas to avoid out-crossing with native species (Barroso et al., 2005). These areas, however, are negligible compared to the remaining available area.

There is a great expectation that the amount of Bt cotton planted in Brazil will increase quickly in the next few seasons, but this could be hindered by the low number of Bt varieties commercially available to address the diverse environments in the different regions. Another problem that may limit the adoption of Bt cotton in Brazil is the occurrence of the boll weevil from mid to late season, which requires a significant number of sprays. Moreover, Bt cotton will still require insecticides to control common nontarget pests such as mites, thrips, stemborers, and several hemipterans: Aleyrodidae, Aphididae, Pentatomidae, Miridae, Cydnidae, and Pyrrochoridae. In addition, cotton varieties susceptible to virus diseases require intense control of aphids, whiteflies, and cicadellids. Nevertheless, based on the pest com-

plex that occurs in each cotton-producing region in Brazil, and with the adoption of cotton varieties with the stacked gene Cry1Ac/Cry2Ab or Cry1F/Cry1Ac (when available), there is likely to be a considerable reduction of insecticide use in Brazil. For the Northeast region, this could drop by at least half from five to six sprays down to three. The remaining sprays will be used mainly to control aphids and boll weevil. In the Midwest, insecticide use could drop from 10-17 applications to 5-10 applications, depending on the selection of resistant varieties or those susceptible to virus diseases and boll weevil infestations (the current average in the Midwest is around 10 sprays per season; Richetti et al., 2004). Bt cotton is likely to have the lowest impact in the Meridian region by removing only two sprays. Most insecticides in this region are used to control heavy infestations of the boll weevil, which are common in this region.

8 Future of Transgenic and Pest Management in Cotton

Productivity with sustainability is the ideal cotton production system envisioned in the future to meet consumer and environmental demands. Obtaining sustainable and profitable yields will require improvements in cultivation practices and genetics, and will vary widely from region to region. One modern approach to improving the productivity of cotton is the development of transgenic varieties that combine novel genes conferring desirable traits—that is, genes that not only facilitate cultivation practices such as insect control and weed management as shown in this review, but also to address market and environmental demands. Production-reducing impacts on environment fauna and flora that can be viewed, measured, and demonstrated will aggregate value in the final product for the market. The adoption of Bt and herbicide transgenic cotton has reduced operational costs and energy input in the system based on a reduction of chemicals and petroleum in machinery. The maximization of revenue does not always require high yields, but instead requires the best fiber quality possible to get the maximum prices per unit yield with low input.

Drought tolerant and higher water-use efficient varieties are areas of great activity in biotech laboratories around the world. Transgenic cotton that is tolerant to drought and soil salinity assures expansion into new regions and soils considered inappropriate or previously cultivated only with heavy irrigation and fertilization. Water availability will dictate agricultural activity in the future for many areas in the world. Cotton is among the annual crops for which low water requirement and improved drought tolerance will be very important for its production in Africa and many other parts of the world with variable rainfall. Yield increases of 5–10% are expected using drought-tolerant varieties. Biotech companies recognize this as the next frontier to be conquered. Similarly, improvement of physiological mechanisms for nitrogen uptake will reduce the dependence on nitrogen fertilizers.

Pest management of insects and weeds with transgenic crops will become more secure with the adoption of varieties expressing other genes than those already marketed. As mentioned above, different modes of action are required to sustain

susceptibility in insects targeted by the proteins expressed in the plants, and new stacked genes, new Bt proteins (e.g., VipCot®), or proteins from other sources such as protease inhibitors will make important contributions to insect resistance management and insect control. Likewise, new cotton varieties tolerant to different herbicides will offer options in herbicide selection aimed at weed resistance management. The current herbicide-tolerant varieties have introduced changes in cultivation methods that were not previously possible on the present scale. Besides improving agronomic aspects of cotton cultivation, new varieties carrying new traits developed by multiple biotech companies will offer growers additional options better suited to their field environments.

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Conservation Agriculture: A Different Approach for Crop Production Through Sustainable Soil and Water Management: A Review

Fabio Stagnari, Solange Ramazzotti and Michele Pisante

Abstract Tillage-based soil management for intensive crop production generally leads to soil degradation and eventual loss of crop productivity. Moreover, farmers have to face high costs for fuel, labor, agro-chemicals, and other production inputs required by intensive cropping. Intensive tillage causes a greater loss of soil carbon and increases greenhouse gas emission, mainly CO_2, that not only impacts soil productive capacity but also impacts atmospheric quality that is responsible for "climate change." This article reviews the practice of conservation agriculture as a viable system for sustainable crop production and agricultural development. The following major points have been found to be associated with the adoption of conservation agriculture when compared with tillage-based agriculture: improved soil structure and stability; increased drainage and water-holding capacity; reduced risk of rainfall runoff and pollution of surface waters with pesticides of up to 100% and fertilizers up to 70%; and about one quarter to one half lower energy consumption and lower CO_2 emissions. Moreover, crop residues are more naturally left on the surface to protect the soil and to drive the carbon cycle towards the conversion of plant biomass carbon to soil organic matter and humus. The changes in the physical environment affect many different groups of organisms, and although there is a wide range of responses among different species, most organism groups are in greater abundance in conservation agriculture than in tillage-based systems. The practice of conservation agriculture requires attention to crop rotation, adequate weed control, management of crop residues, mulching, introduction and management of cover crops, changes in seeding, and transplanting equipment. Despite the benefits linked to the practice of conservation agriculture, there is still much scepticism—especially in Europe—about the suitability of the conservation practice within the European soil and climatic conditions and cropping systems. Nevertheless, it will be more necessary than ever for farmers to adopt sustainable agricultural systems that can

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simultaneously meet their economic needs, address the concerns of consumers, and minimize the impact on the environment.

Keywords Sustainability · Conservation agriculture · No-till · Soil erosion · Soil organic matter

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1 Introduction

During the second half of the 20th century, many energy-consuming agricultural practices were adopted as part of the modern scientific approach to achieve higher yields. Such practices were also encouraged by the large availability of cheap fuel. Heavy tillage, frequent weed control, abundant fertilization and surface water movement across large fields by pumping were keystones of the dominant production paradigm. Plow-based soil cultivation, in particular, has become so common in mainstream modern agriculture that the term “tillage” is widely used as a synonym for “agriculture” (Dick and Durkalski, 1997). Nevertheless, continuous soil disturbance through cultivation and particularly through soil inversion has lead to the degradation of soil structure, soil compaction, and decreased levels of organic matter in soil. This, in turn, has caused a wide range of environmental impacts, including soil degradation, water and wind erosion, eutrophication, increased carbon emissions released from the soil due to the use of high energy-consuming machinery, and an overall reduction in beneficial soil organisms and mammals. As soil accumulated over the eons, it provided a medium in which plants could grow. In turn, plants protected the soil from erosion. The agricultural activity of humans has been disrupting this relationship. Climate change has also exacerbated the problems of degradation and variability as rainfall events have become more erratic with a greater frequency of storms (Osborn et al., 2000). A way of minimizing these negative impacts on the agricultural environment is offered by a recently-promoted approach to agricultural

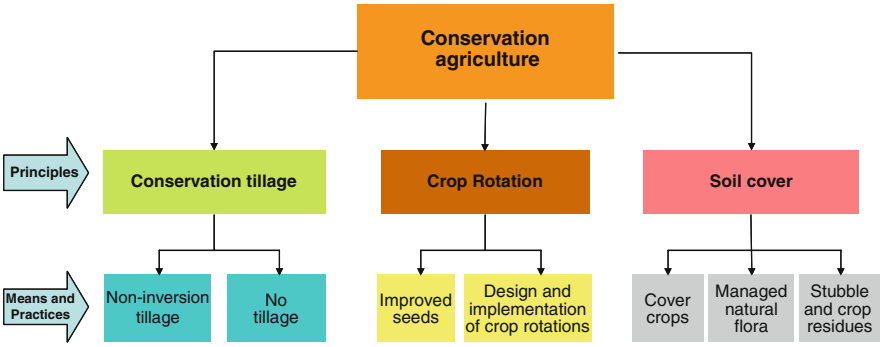

Fig. 1 The three principles of conservation agriculture and the main practices and means needed to achieve each principle

production, the "conservation agriculture" (CA), defined by Food and Agriculture Organization (FAO; CA website, 2004) as a system based on minimal soil disturbance (no-till, minimum tillage) and permanent soil cover (mulch, crop residue) combined with diversified rotations with legumes (Fig. 1).

Indeed, CA is the generic title for a set of farming practices designed to enhance the sustainability of food and agriculture production by conserving and protecting the available soil (Hubbard et al., 1994; Karlen et al., 1994), water, and biological resources such that the need for external inputs can be kept minimal (Garcia-Torres et al., 2003). Its primary feature, and indeed a central tenet, is the maintenance of a permanent or semipermanent soil cover—be it a live crop or dead mulch—that serves to protect the soil from sun, rain, and wind, and to feed soil biota.

Different labels such as "organic farming" and "conservation tillage" have been used for highlighting a specific difference from "modern" industrial agriculture, emphasizing some aspects of CA. In the case of organic farming, although biodiversity, biological cycles, and soil biological activity are features in common with CA, the minimal use of off-farm inputs does not match with the principles of CA. During the transition phase, especially, the loss of pest and disease maintenance previously afforded by conventional tillage necessitates chemical inputs that ideally are used in moderation as part of an integrated pest management system to ensure a healthy biotic community. This biotic community is essential as it provides a "biological tillage" that serves to replace the functions of conventional tillage. Hence, although CA more deliberately exploits natural processes than modern plow-based agriculture, it is not synonymous with organic agriculture. The "conservation tillage" label refers to a set of practices adopted by modern plow-based conventional tillage to enhance water infiltration and reduce erosion risk. This term is commonly applied to no-tillage, direct drilling, or minimum tillage practice when it is associated with a cover of crop residues on at least 30% of the soil surface, and associated with some conservation goals such as the conservation of time, fuel, earthworms, soil water, and nutrients (Baker et al., 2002). Because conservation tillage still depends on tillage as the structure-forming element in the soil, conservation tillage practices can be a transition step towards CA agriculture (Fig. 1).

According to the FAO definition, CA aims to conserve, improve, and make more efficient use of natural resources through integrated management of available soil, water, and biological resources combined with external inputs. CA contributes to environmental conservation as well as to enhanced and sustained agricultural production, and it can also be referred to as "resource efficient" or "resource effective" agriculture (Garcia-Torres et al., 2003). In fact, CA is at the base of the resource conservation technologies (RCT) that have been shown to improve yields while reducing water, nutrient, and energy consumption and lowering the impact on environmental quality.

CA emphasizes that the soil is a living system, essential to sustaining the quality of life on our planet. The principles of CA and the activities to be supported are described as follows:

- Maintaining permanent soil cover and promoting minimal mechanical disturbance of soil through zero tillage systems to ensure sufficient living and/or residual biomass to enhance soil and water conservation and control soil erosion.
- Promoting a healthy, living soil through crop rotations including legumes, cover crops, and the use of integrated pest management technologies, thus making more efficient use of fertilizers, pesticides, herbicides, and fungicides by matching them to crop needs.

Table 1 Benefits and costs associated with conservation agriculture

Advantages	Drawbacks
Reduction in on-farm costs: savings of time, labor, fuel, and machinery	Purchase of specialized planting equipment
Increase in soil fertility and moisture retention, resulting in long-term yield increase, decreasing yield variations, and greater food security	Short-term pest problems due to the change in crop management
Stabilization of soil and protection from erosion leading to reduced downstream sedimentation	Acquiring of new management skills
Reduction in toxic contamination of surface water and groundwater	Application of additional herbicides
More regular river flows, reduced flooding, and the re-emergence of dried wells	Formation and operation of farmers' groups
Recharge of aquifers as a result of better infiltration	High perceived risk to farmers because of technological uncertainty
Reduction in air pollution resulting from soil tillage machinery	Development of appropriate technical packages and training programs
Reduction of CO_2 emissions into the atmosphere (carbon sequestration)	
Conservation of terrestrial and soil-based biodiversity	

Source: adapted from ECAF (European Conservation Agriculture Federation, 2001), and FAO (2001).

Integration of these fundamental principles enhances the development and functionality of crops' root systems as a consequence of an increased depth and more regular water and nutrient uptake. In particular, the advantages (Table 1) of the model proposed by CA lie in a greater water infiltration rate associated with improved soil porosity and an associated lower erosion rate (Unger, 1991), greater water retention (Angulo-Jaramillo et al., 2000), higher organic matter content (Lal and Kimble, 1997), improved soil biological fertility and an abundance of soil micro-organisms (Epperlein, 2001), enhanced soil structure (Tebrügge and Düring, 1999), lower mechanical and labor costs, and higher net returns (Knowler and Bradshaw, 2007).

The main goals of the present review are to describe the agronomic and environmental aspects of CA, its adoption for annual crops and orchards, and its spread worldwide.

2 Agronomic and Environmental Aspects of Conservation Agriculture

2.1 Soil Protection

Soil quality has been defined as the capacity of the soil to function within ecosystem boundaries to sustain biological productivity, to maintain environmental quality, to promote plant and animal health (Doran and Parkin, 1994), and to sustain crop growth and yield. Agricultural activity can reduce soil quality, especially tillage, which gives rise to processes that may damage the natural soil ecosystem (Pisante, 2007). Plow-based tillage determines soil compaction—especially when repeated passes of a tractor are made to prepare a seed bed or to maintain a clean fallow (Hobbs et al., 2008)—and destroys the original soil structure, breaking up the macroaggregates into microaggregates (Angers et al., 1992), thereby altering many physical proprieties. These include the stability of aggregates > 2 mm (Chan, 2001), which is widely recognized as key indicator (Nael et al., 2004) of soil quality, pore space and size distribution, water holding capacity, and soil water content. This leads to increased runoff and poor infiltration (Hussain et al., 1999; Ferreras et al., 2000; Raper et al., 2000). Soil structure is important for all crops. It regulates soil aeration and gaseous exchange rates, the movement and storage of water (Abu-Sharar and Salameh, 1996), soil temperature, root penetration and development, nutrient cycling and resistance to structural degradation, and soil erosion (Barthès and Roose 2002). Soils with good structure have a high porosity between and within aggregates, but soils with poor structure may not have macropores and coarse micropores within the large clods, restricting their drainage and aeration (Shepherd et al., 2008). As oxygen depletion increases, orange and ultimately grey mottles are formed. Poor aeration reduces the uptake of water by plants and can induce wilting. It can also reduce the uptake of plant nutrients, particularly nitrogen, phosphorus, potassium, and sulphur. Poor aeration also retards the breakdown of organic residues, and can cause chemical reactions that are toxic to plant roots (Shepherd et al., 2008). The

presence of soil pores also enables the development and proliferation of superficial roots throughout the soil in the rooting zone. Roots are unable to penetrate and grow through firm, tight, compacted soils, severely restricting the ability of the plant to utilize available water and nutrients, and also reducing fertilizer efficiency and increasing the susceptibility of the plant to root diseases.

Soils managed according to CA principles show significantly decreased bulk density at the surface. This results from the exiting mulch layer on top of non-tilled soils (Beisecker, 1994) that provides organic matter and food for soil fauna, which loosens surface soil as a result of burrowing activities. Also, below the subsurface layer (25±30 cm soil depth), the bulk density of the non-tilled soils usually is lower than in tilled soils (Tebrügge and Düring 1999). No-tillage also dramatically reduces the number of passes over the land and thus compaction; the FAO now includes "controlling in-field traffic" as a component of CA. This is accomplished by having field traffic follow permanent tracks that can be combined with a permanent bed planting system rather than planting on the flat (Sayre and Hobbs, 2004).

In erosion-prone environments, whether in wet or dry warm zones, inversion of soil through tillage promotes unnecessary moisture loss; at the same time, the crop residues that should protect soil from erosion by wind or water and slow soil moisture loss after rain are buried (Pisante, 2002). Soil erosion represents a threat for the sustainability of agriculture worldwide (Laflen and Roose, 1998), and in Europe is considered to be one of the most important environmental problems (Fig. 2). Despite the economic impact on a world scale, the severity of soil erosion is difficult

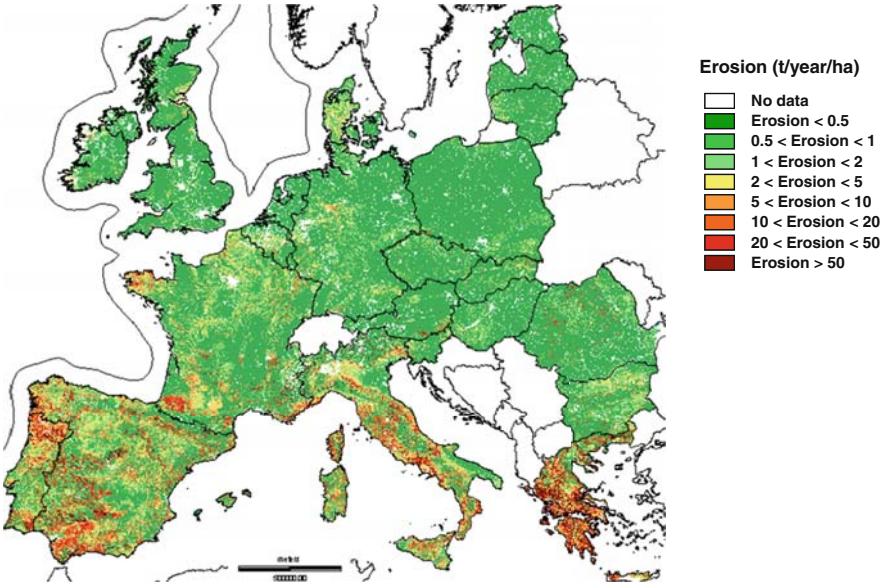

Fig. 2 Incidence of soil erosion in Europe

to estimate. Lal (2001) reported $37.6 billion (US) as the annual social costs. In the United States, Pimentel et al. (1995) estimated the erosion costs to be 85.5 € ha^{-1}. Recently, a study carried out in 13 European member states reported that soil degradation determined significant annual social costs quantified in 0.7–14 billion € for erosion and 3.4–5.6 billion € for organic matter loss.

Intensive plow-based tillage also represents a major cause of organic matter depletion (Six et al. 2000) by exposing physically protected organic material (Paustian et al., 1997), increasing oxidation over time, and leading to loss of soil biological fertility and resilience (Lal 1994). Long periods of annual cropping with intensive tillage and without cover crops are recognized to decrease soil organic matter (Feller et al., 1996). This is particularly important in the tropics where organic matter reduction is processed more quickly with low carbon levels, resulting in damage after only one or two decades of intensive soil tillage (Campbell et al., 1996). Tillage also may alter SOM dynamics by changing its position within the soil matrix, either by releasing organic material from within aggregates during disruption, or occluding materials during the aggregates' formation (Plante and McGill, 2002). The decline of SOM reduces the fertility and nutrient-supplying potential of the soil so that the nutrient requirements by crops from artificial sources increase, and other major and minor elements are more readily leached. This results in an increased dependency on fertilizer input to maintain nutrient status.

The adoption of CA involves minimal soil disturbance (due to the use of no-tillage and direct seeding), management of cover crops, crop residues, and crop rotation—all of which preserve SOM content (Wright and Hons 2004; Thierfelder et al., 2005). Cover crops may influence soil aggregation and associated Carbon (C) and Nitrogen (N) pools, thereby affecting soil quality and productivity (Sainju et al., 2003; Holeplass et al., 2004). Conservation agriculture practices, as reported by Reicosky et al. (1995), increase SOM content following the accumulation rate from 0 to 1.15 t C ha^{-1} per year with the highest values in temperate climatic conditions. Similar data have been obtained by other studies (Lal et al., 1998) showing an accumulation rate for organic C ranging from 0.1 to 0.5 t ha^{-1} per year. This aspect is extremely important due to the multiple role played by the organic matter in the soil. It regulates most biological, physical, and chemical processes that collectively determine soil health. It promotes infiltration and water retention, it helps to develop and stabilize soil structure and mitigate the impact of wheel traffic and cultivators, and reduces the potential for wind and water erosion. Organic matter is also an important source of C (Fig. 3), and a major reservoir for plant nutrients.

The presence of surface cover helps to prevent erosion and compaction by minimizing the dispersion of the soil surface by rain or irrigation. It also helps to reduce crusting by intercepting the large rain droplets before they can strike and compact the soil surface (Dormaar and Carefoot, 1996). It further serves to act as a sponge, retaining rain water long enough for it to infiltrate into the soil. Crop residue management improves aggregate stability (Sonnleitner et al., 2003), and this leads to reduced soil detachment and improved infiltration rates (Ekwue, 1992).

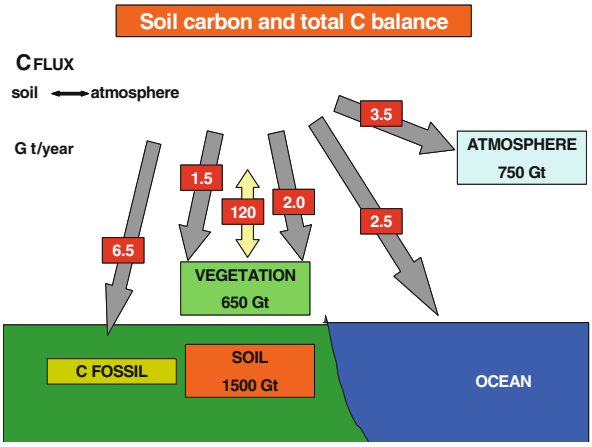

Fig. 3 C cycle on earth: soil C and total C balance (International Geosphere Biosphere Program, 1998)

2.2 Water Protection

Agriculture is considered to be the largest user of freshwater on a global basis and one of the major causes of surface and groundwater resource degradation through erosion and chemical run-off. In some circumstances, soil sediments represent the main contaminants of water flows. In Germany, simulated rainfall (63 mm h^{-1} for one hour) on a silty soil managed with plow-based cultivation (plow+secondary tillage) caused sediment losses of 6,400 kg ha^{-1} (Tebrügge and Düring, 1999). Quine and Walling (1993) estimated that 27–86% of eroding sediment leaves the field (Quine and Walling, 1993) as consequence of soil erosion. Associated with this movement of soil and water are agrochemicals, pathogens, organic matter, and heavy metals (Christensen et al., 1995), all of which have been frequently found to damage the water ecosystem (Uri et. al., 1998). Sediments have been shown to cause sublethal and lethal responses in freshwater fish, invertebrates, and periphyton (Alabaster and Lloyd, 1980; Newcombe and MacDonald, 1991). A direct consequence of leaching of inorganic fertilizers, organic matter, and pesticides into the water is the eutrophication phenomena widespread throughout the world where an industrialized approach to modern farming is practiced (Harper, 1992).

The role of CA in reducing the risk of these pollutants reaching surface and ground water is well understood (Logan, 1993). In the United States, CA was shown to reduce run-off by between 15 and 89%, and reduce dissolved pesticides, nutrients, and sediments within it (Fawcett 1995; Clausen et al., 1996). In a 15-year study comparing different CA techniques, sediment loss was 1,152 and 532.82 kg ha^{-1} per year for chisel-plow and disk vs. not tilled, respectively (Owens et al., 2002). Plow-based cultivation over time affects the rate and proportion of rainfall infiltration, and thereby groundwater recharge, river flow rates, and the need for irrigation (Harrod, 1994; Evans, 1996). In areas of low rainfall, CA helps retain water in the upper soil layers (Rasmussen 1999). In those circumstances, direct drilling combined with

Table 2 Effect of tillage on water quality and diffuse pollution (Source: Jordan et al. 2000)

Measurements	Plow	Non-inversion tillage	Benefit compared to plowing
Runoff (l ha^{-1})	213.3	110.3	48% reduction
Sediment loss (kg ha^{-1})	2,045	649	68% reduction
Total P loss (kg P ha^{-1})	2.2	0.4	81% reduction
Available P loss	3 x 10^{-2}	8 x 10^{-3}	73% reduction
Organic Nitrogen (mg N s^{-1})	1.28	0.08	94% reduction
Soluble phosphate (μg P s^{-1})	0.72	0.16	78% reduction
Isoproturon (μg s^{-1})	0.011	Not detected	100% reduction

Comparison of herbicide and nutrient emission from 1991 to 1993 on a silty clay loam soil. Plot 12 m wide were established and sown with winter oats in 1991, followed by winter wheat and beans.

stubble retention was shown to increase rain infiltration, leading to a reduced run-off compared with cultivated soil (Carter and Steed, 1992).

The effect of tillage on the leaching of pesticides was reviewed by Rose and Carter (2003), and although they concluded, as did Flury (1996), that soil cultivation was an important determinant of pesticide-leaching losses, the effect of adopting CA was highly variable. CA can have a negative impact during the first year of transition due to the usage of a herbicide for grass weed control (Elliot and Coleman, 1988) when it is applied shortly before a heavy rainstorm and then washed directly into the pores (Kanwar et al., 1997; Ogden et al., 1999). On the other hand, the presence of earthworms can cause higher amounts of organic matter that retains agrochemicals, and therefore help prevent the movement of pesticides (Sadeghi and Isensee, 1997; Stehouwer et al., 1994). Studies evaluating the reduced risk of pesticide contamination in surface waters due to adoption of CA have only been conducted in the United States. Direct drilling reduced herbicide run-off by 70–100% (Fawcett, 1995), and leaching of isoproturon was reduced by 100% within a three-year period of CA (Table 2) (Jordan et al., 2000).

2.3 Air Protection

During the last 100 years, the average temperatures in Europe have increased 0.95°C and will probably rise 2–6°C within the next century (EEA, 2005). The high CO_2 emissions discharged into the atmosphere due to the use of fossils fuels represent the main contribution to global warming. Agriculture contributes to CO_2 emissions as an energy-demanding process in the production of arable crops, as energy utilization in agrochemicals manufacturing, transportation, application, and the break-down of soil organic matter (SOM). With regard to the latter, soil represents the widest C-releasing surface—about 1,500 Gt per year—equivalent to almost three times the C amount accumulated in the biomass, and two times the C present in the atmosphere (Fig. 3). Thus, in agricultural systems, any modification in soil management induces changes in the overall C stock (Six et al. 2002).

Adoption of CA can help reduce CO_2 emissions in many ways. Noninversion soil cultivation methods (direct drill, disc + drill) and, in general, minimal mechanical soil disturbance clearly has a lower energy usage than systems based on plowing (Leake, 2000).

The use of fertilizers can be reduced with CA due to residue management and cover cropping, which guarantee an improved nutrient recycling (Lal et al., 1999; Carter, 1993) and soil microbial activity. By promoting SOM-building, CA can significantly reduce CO_2 emissions (West and Marland, 2002). There is evidence of higher amounts of C in the soil where CA was applied in comparison to conventional plow-based soil management: 8% higher, equivalent to 285 g SOM/m^2 in the UK (Holland 2004); and 0.5% higher using an integrated approach over 19 years of study in the Netherlands (Kooistra et al., 1989). In a long-term, plow-based tillage study at Drabble (Buenos Aires, Argentina), SOM in the 0–30 cm layer of a loamy soil under six years of continuous row cropping, diminished 19% with mouldboard plowing, 7% with chisel plowing, and 0.4% with no-till (Diaz-Zorita, 1999). Increases in SOM in the upper surface layers were also found in a number of studies conducted in Scandinavia (Rasmussen, 1999), when CA principles where applied. Lindstrom et al. (1998) report a potential C accumulation of 0.1–1.3 t ha^{-1} per year by adopting CA, while intensive cultivation techniques strongly reduced C levels (Triberti et al., 2004). In this way, the soil can play an important role as a "carbon sequestration sink," stabilizing the CO_2 concentration in the atmosphere (Bernoux et al., 2006). In total, methods of CA-based soil management have been estimated to save 23.8 kg C ha^{-1} per year (Kern and Johnson 1993).

2.4 Biodiversity

The importance of soil biodiversity in agriculture has not been appropriately considered because crop productivity has been increased through the use of inorganic fertilizers, pesticides, plant breeding, soil tillage, liming, and irrigation. Soil biodiversity is normally indicated as the variability of the living forms, soil fauna, flora, vertebrates, birds, and mammals within a habitat or a management system of a territory involved in agricultural activity.

With regard to soil fauna, microbial mass diversity and biological activity are higher in undisturbed soil or soil system that is managed using CA techniques compared to those receiving deep cultivation (Nsabimana et al., 2004; Spedding et al., 2004). With respect to microfauna, Cochran et al. (1994) suggest that management practices that favor bacteria would also be expected to favor protozoa, since bacteria are their main food source. Also, the abundance of mesofauna (in particular, potworm) was greater where CA was practiced in comparison to compacted soil (Röhrig et al., 1998). The negative effects on microarthropod populations are caused, in part, by the physical disturbance of the soil from plow-based tillage. Some individuals may be killed initially by abrasion during tillage operation, or by being trapped in soil clods after tillage inversion (Wardle, 1995). One of the

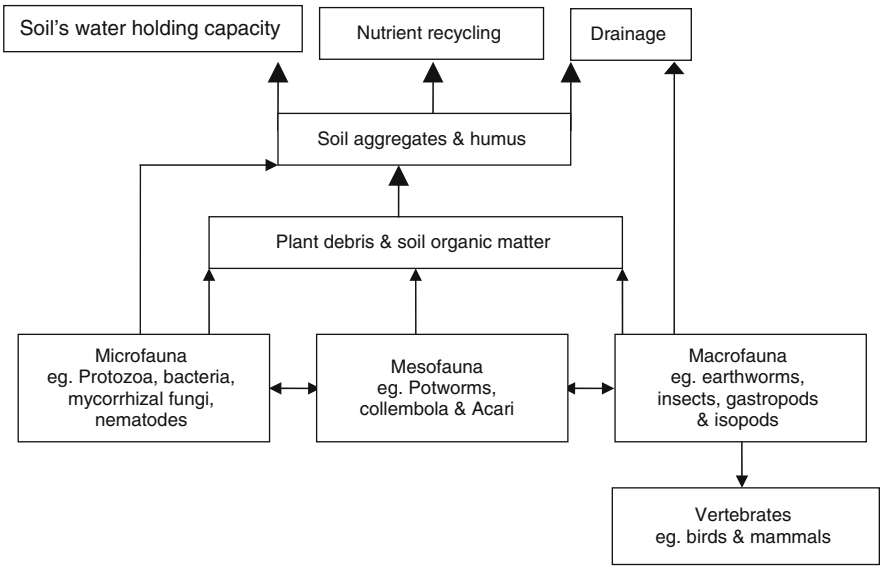

Fig. 4 Interactions between soil-associated fauna and soil dynamics

main groups within the mesofauna are the enchytraeids, which can increase aeration, water infiltration, and root growth because of their burrowing habit (Cochran et al., 1994). They may be either inhibited or stimulated by tillage: the apparent stimulation, which is in contrast with most of the other groups, may be due to their ability to recover from disturbances (Wardle, 1995) (Fig. 4).

Earthworms are a significant part of the macrofauna in many soils, affecting soil properties through their feeding, casting, and burrowing activities. They can modify the soil's physical structure, decreasing the risk of erosion (Arden-Clarke and Hodges, 1987). Minimum soil disturbance, especially if combined with the return of crop residues and additional organic manure supply, nearly always increases earthworm populations (Kladivko, 2001), especially deep-burrowing species (Edwards and Lofty 1982). Numerous examples from research plots (Barnes and Ellis, 1979; Edwards and Lofty, 1982; House and Parmelee, 1985) support the conclusion that reduction of tillage intensity encourages earthworm populations (Table 3). Moldboard plowing and no-till represent the two extremes of agricultural soil management systems, and systems with intermediate levels of soil disturbance and surface residue usually show populations to be similarly intermediate between the two extremes. Earthworms are also important for mixing plant residues and other materials into the soil, which may be particularly important in no-till systems due to the lack of mechanical mixing by tillage implements (House and Parmelee, 1985). The same has been found for gastropods, isopods, and myriapods. Results from many studies conducted in North America and in Europe on the effects of CA on arthropods abundance are inconsistent, showing an increase (Andersen, 1999; Holland

Table 3 Abundance of earthworms (number m^{-2}) under no-tillage, conventional tillage, and permanent pasture

No-till	Conventional	Pasture	Remarks	Reference
270	90	–	On a very poorly drained soil, cultivation by normal plowing	Boone et al. (1976)
137	67	–	Cultivation by deep plowing	Gerard and Hay (1979)
913	213	–	Cultivation involved mouldboard plowing, 3 disk plowing and 2 rotary tilling	House (1985)
342	130	–	Lupin/wheat rotation, three cultivation to 7 cm with a duck food scarifier	Rovira et al. (1987)
275	117	–	Cultivation involved scarifying (10 cm) 2 or 3 times and a light harrowing (7 cm)	Haines and Uren (1990)
266	48	477	–	Deibert et al. (1991)
467	52	1,017	–	Springett (1992)
250	175	825	Lismore site, after 8 years of cropping	Francis and Knight (1993)
–	52	168	–	Mele and Carter (1999)

and Reynolds, 2003), decrease (Andersen, 1999; Holland and Reynolds, 2003), or no effect (Huusela-Veistola, 1996; Holland and Reynolds, 2003). This is probably due to the fact that research has often been done on "no permanent no-till," "no-till without residues," and "rotation or conservation tillage."

Summarizing the effects of no-tillage on soil fauna, Kladivko (2001) concluded that the larger species are more vulnerable to soil cultivation than the smaller. A review of 45 studies of tillage and invertebrate pests (Stinner and House, 1990) showed that for the investigated species, 28% increased with decreasing tillage, 29% did not change with a tillage system, and 43% decreased with decreasing tillage. Beetles (Coleoptera) and spiders (Araneae) are important members of the macrofauna that are usually much reduced by plow-based tillage operations (Wardle, 1995). Reduced populations under conventional tillage are likely due, in part, to physical disturbance and abrasion from the tillage operation itself, but the reduction in surface residue cover is probably more significant.

3 Conservation Agriculture Adoption in Annual Crops and Orchards

For many centuries, most agricultural systems worldwide have been based on multicropping, which on the same farm contemporaneously accounted for herbaceous annual and perennial species and woody plants. The industrialization of agriculture

led to a specialization of farming, reducing the number of cultivated species at the farm level, and to monocropping on a large scale (Bonciarelli, 1987). This initially was positive because it led to higher yields and subsequently increased the income potential of farm lands. Subsequently, unexpected problems have arisen in terms of difficult-to-control diseases and weeds; however, the most negative consequence has been connected to the deep plowing repeated every year. In particular, the rapid depletion of organic matter has led to a reduction of soil fertility and, in some cases, even to desertification. On this basis, there has been a growing interest in the need to preserve soil fertility and therefore productivity. Thus, CA practices represent ever more common alternatives oriented to optimize farming resources through the reduction of external inputs (Garcia-Torres et al., 2003).

3.1 Annual Crops

Adoption of CA techniques in annual crops has been increasing in the last few years, although in the past it faced some resistance because of management limitations. It was the case of weed control (Koskinen and McWhorter, 1986) and lack of proper sowing machines in wheat (Logan et al., 1987); suitable seed bed and correct pest control management in canola (Pisante, 2007); and a lack of suitable hybrids, tools, and machineries, and a scheduled program of weed and pest management in maize (Pisante, 2007). Difficulties were also encountered when transplanting vegetable crops, with results being unsatisfactory at times (Rutledge and Dutton, 1999), especially in compacted and dry soil where, due to low adherence between soil and seed or transplant, plant survival was severely constrained (Morse 1999). Furthermore, on a no-tilled soil, more difficulties can arise in weed control (Standifer and Best, 1985; Lanini et al., 1989), although in some cropping systems, achievement in weed management has allowed satisfactory control, as in the case of rice-wheat biennial rotation in South Asia (Gupta and Seth, 2007).

For a correct application of CA to annual crops, several aspects that can be affected by soil management techniques have to be considered. Among those particularly important are soil type, genotype, rainfall, and soil water retention capacity (Boone, 1988; Lampurlanes et al., 2002; Hemmat and Eskandari, 2004). In this respect, controversial results have been collected in several studies regarding CA adoption: Chastain et al. (1995) reported that wheat yield and growth are not influenced by mulching with plant residues in humid environments. On the contrary, increase in wheat yield has been observed in other studies and related to higher soil water content (Rao and Dao, 1996; Papendick and Miller, 1977), evapotranspiration fluxes lower soil temperatures (Gauer et al., 1982), and higher water infiltration rates into the soil (Good and Smika, 1978, Unger and McCalla, 1980; Allmaras and Dowdy, 1985). Papendick and Miller (1977) stated that, in the presence of mulching with straw, wheat yield can increase by 20% due to an additional 20 mm of rainwater in a low rainfall area. In some circumstances, the excessive size and concentration of crop residues could reduce the adhesion between soil and seed with lower

Fig. 5 Wheat at tilling, with abundant crop residues on soil surface (Source: Michele Pisante)

seedling emergence as a consequence (Acharya et al., 1998). However, this problem can be easily solved by cutting finer residues and spreading (Fig. 5). In Italy, a 12-year study (Pisante, 2007) comparing plow-based systems to no-till systems in durum wheat has showed that the average yields were not different between the two systems (Fig. 6), although higher yield and water use efficiency occurred in a no-till system when precipitations were < 300 mm during the crop cycle. This is supported by De Vita et al. (2007), who found similar results for durum wheat over a three-year period (2000–2002) at two locations (Foggia and Vasto) in southern Italy.

Summarizing the effect of tillage practices on sorghum yield at six experimental sites located in the subhumid and semi-arid Pampas region, Buschiazzo et al. (1999) concluded that average yields with no-till and reduced-till were higher than with conventional tillage (mouldboard-till). No-till double-cropped soybean yields are generally higher and more stable between seasons (Ferrari, 1997). In Argentina, where no-till was initiated after four years of tillage, lower first-year corn yields were observed for no-till than for chisel-till. But in the following seasons, no significant differences in sunflower or maize yield were observed between tillage practices, and after four years, corn yields were greater for no-till than for chisel-till. The CA system reduces the energy input into maize and soybean management in Argentina, and similar behavior has been described in other countries—e.g., Australia, New Zealand, and the United States (Frengley, 1983; Thomas, 1985; Cornish and Pratley, 1991). In the case of winter oil seed rape (Brassica sp.) on an average of different soil types in Sweden, yields after direct drilling were only slightly less than drilling on a seed bed prepared with conventional tillage-based agronomic practices (Cedell, 1988). Direct drilling of potatoes (*Solanum tuberosum* L.) may be a realistic alternative to the plow-tillage planting method. In Norway, satisfactory yields were obtained in late harvested potatoes directly planted into not-cultivated soil (Ekeberg and Riley, 1996).

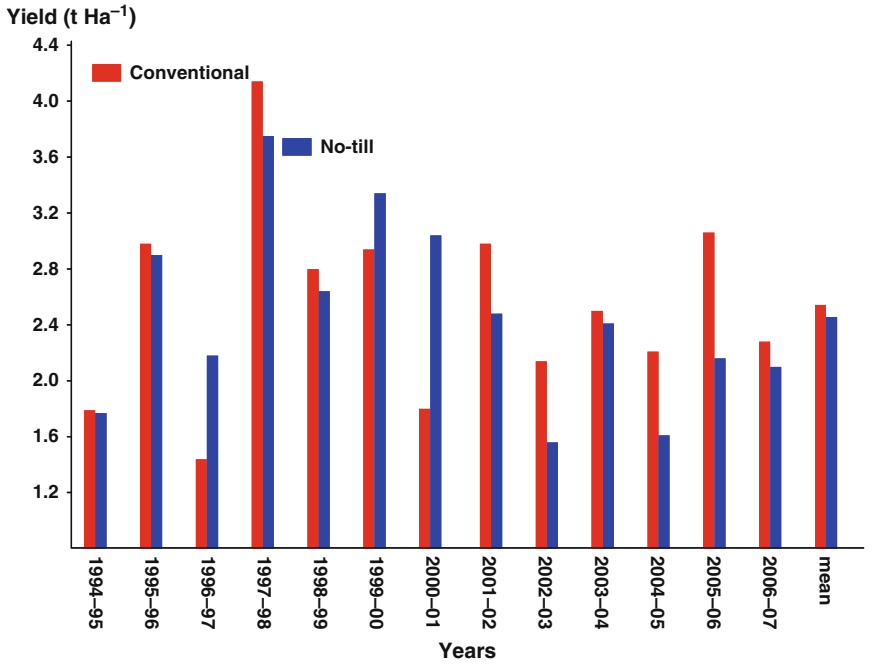

Fig. 6 Yields of wheat in monocropping from 1995 to 2007 at Foggia (Southern Italy) in conventional and no-tillage systems (Source: Pisante, 2007)

To reduce machine transit, and hence soil compaction, fertilizers can be applied at the time of seedbed preparation or seeding. In this way, fertilizers can be positioned at a depth suitable for good crop root development.

In the CA adoption on annual crops, a critical is pest competition, and weeds (Bilalis et al., 2003; Koskinen and McWhorter, 1986) because of initially higher seed accumulation on the soil surface due to reduced tillage (Wruckle and Arnold, 1985). However, weed management has to be adapted to the requirements of each cultivation area. For instance, on the Indo-Gangetic Plains, the use of no-tillage for wheat planting in conjunction with a new herbicide provided effective weed control at lower rates (Mehla et al., 2000), especially when closer row spacing was adopted (Ali and Tunio, 2002). Hence with CA, a more efficient chemical weed control is required to be integrated with an indirect weed control system such as proper crop rotation, herbicide rotation, crop plant competition (cover crops), variety choice, and the proper management of fertilizer and water. In this way, less environmental impact and reduced costs can be achieved with respect to the only chemical weed control.

3.2 Orchards

Although many different trellis systems are used in tree crops, they can all be suitable for CA. Good soil management practices are needed to maintain good growth

conditions and productivity to safeguard the functionality of the tree, especially during the crucial periods of plant development and fructification (Pool et al., 1990). To achieve this, management practices need to maintain and promote the condition, and therefore functionality, of the soil, particularly with regard to its aeration status and the supply of nutrients and water to the plant (Merwin, 2004). To this end, the soil needs to have an adequate soil structure to allow an effective root system to maximize the utilization of water and nutrients and to provide sufficient anchorage for the plant. Good soil structure also promotes infiltration and movement of water through the soil, minimizing surface ponding, run-off, and soil erosion. Indeed, most orchards are mixed systems where trees on the rows coexist with herbaceous plants along the alleys. The establishment of cover crops in orchards is the most suitable soil management practice to protect the soil surface from erosion, to preserve the environment, to reduce production costs, and to enhance the quality of the fruit. Cover cropping not only helps in reducing water run-off and soil erosion, but also improves the physical characteristics of the soil, enriches soil organic matter content and soil life (including earthworm numbers), and suppresses soil-borne disease by increasing microorganism biodiversity. Cover crops, on the other hand, can compete for minerals, water, and fertilizer if they are not well managed. Where water competition is not limiting (over 700 mm per year with regular distribution), ground cover grass has been used as a soil management system in many orchards. Grass cover is usually limited to the inter-row area, but in some periods (the humid season) it can also be extended to the row. In this condition, it can also represent an agronomic tool to reduce the excessive vigor of the trees (Wheeler et al., 2005; Intrieri et al., 2004). In the absence of irrigation during the hottest months, competition for water could occur during flowering, fruit formation, and development, limiting the final yield. To avoid this competition, a temporary cover crop or natural vegetation can be grown from early autumn to mid-spring, which is often the wettest period, and can be controlled during the hottest period by herbicide application or mowing 2–3 times during the period of major nutrient demand (Fig. 7). Different

Fig. 7 Grass cover on the inter-row area and chemical weed control along the row in a apple orchard (Source: Massimo Tagliavini)

mixes of cover crops, including leguminous species, should be evaluated in different areas (Gan and Goddard, 2008). Where soils have low fertility, legumes species can be introduced into the herbaceous mix to supply nitrogen required by the trees. In the Mediterranean and many temperate regions, positive results can be obtained with self-seeding legumes, such as *Trifolium subterraneum* in subacid soils and *Trifolium brachicalicinum* in clay and basic soils, which can germinate as autumn rain starts growing during winter time and end the crop cycle in early spring leaving their dried residues (Santilocchi and Talamucci, 1999). Furthermore these species avoid the need for replanting, thus minimizing soil disturbance. In addition to legumes, the mix could comprise annual or perennial species, grasses, and other broadleaf plants. Winter annuals can be grown to protect the soil from erosion during the winter and improve the soil's ability to resist compaction when wet. Grasses, with their fibrous root system, are better for improving soil structure and generally add more organic matter than legumes. If allowed to seed in early summer, a seed bank for subsequent regeneration is built up. As an alternative method, herbicide applications before the hot period following natural weed cover could be satisfactory in limiting,

Fig. 8 Mulching with cereal straw in vineyard (Source: Cesare Intrieri)

principally, competition for water (Benites et al., 2005). Moreover, cover crops have a catchment function by increasing nutrient retention such as mineral nitrogen, reducing leaching and water-table pollution (Parkinson, 1993; Merwin et al., 1996).

Within CA practices suitable for orchards, mulching represents an effective tool for soil protection and weeds control, although efficacy and longevity depend on the mulching material. Plastic film applied along the row was particular common in the 1980s (Duncan et al., 1992). Over the last few years, its usage was abandoned because of its easy desegregation under solar radiation or machine traction, and difficult disposal. This has been replaced by organic materials such as compost, bark chips, cereal straw, wine-making residues, grass clippings (spread during mowing), etc.—which, besides mulching effect, can release nutrients enhancing soil fertility (Fig. 8). The application of organic mulches along the row shades the soil, thus reducing temperature and soil evaporation during the summer. Mulches also encourage biological activity (Shengrui et al., 2005), especially earthworms. They suppress weeds and prevent the breakdown of the soil structure under the impact of rain, enhancing water infiltration. Because of their fast decomposition, they need to be replaced nearly every growing season. Pruning canes of the trees contributes to an increase in the soil organic matter, but does not have a significant mulching effect.

4 The Diffusion of Conservation Agriculture

The adoption of CA has not been fast, even in developed nations with good agricultural extension services and well-educated farmers. This is most likely due to the fact that farmers are always attracted by short-term solutions and immediate benefits, while the full technical and economic advantages of CA can be seen only in the medium- to long-term run, when its principles (no-tillage, permanent cover crop, and crop rotation) are well-established within the farming system. It is estimated that the diffusion of CA has reached a surface of 95 million ha worldwide (Derpsch, 2008). Approximately 47% of the technology is practiced in Latin America (in Argentina and Brazil the surfaces have quickly grown from 1 million hectares in the early 90's to the current 18-23 million of hectares), 39% in the United States and Canada, 9% in Australia, and 3.9% in the rest of the world, including Europe (Table 4), Africa, and Asia (Table 5). Although the United States has the biggest area under CA worldwide, it is interesting to note that no-tillage accounts for about 22.6% of all cropland in this country. In Europe, early adoption of CA was voluntary and driven by the need to reduce crop-establishment costs. Farmers did not identify soil erosion or degradation as a major concern (Kuipers, 1970). There is still much scepticism in Europe about the suitability of CA for our climate conditions and cropping system. The wide variety of soil types, perceived high costs of no-tillage equipment, and the intensive hands-on management needed are among the main criticisms of the CA system. Agronomic issues surrounding weed, pest, and

Table 4 Estimation of surface under conservation agriculture in different European countries. Data obtained from ECAF (European Conservation Agriculture Federation) National Associations 2005/2006

Country	Minimum tillage (1,000 ha)	No-till(1,000 ha)	Cover crops inperennial woody crops(1,000 ha)	Total surface CA (1,000 ha)	% NT/arableland	% CA/arableland
Belgium	140	n.d.	n.d.	140		17.2
Denmark	230	n.d.	n.d.	230		10.1
Finland	1,000	150	n.d.	1,150	6.8	52.3
France	3,750	120	n.d.	3,870	0.7	21.1
Germany	2,300	200	n.d.	2,500	1.7	21.2
Greece	230	200	n.d.	430	7.4	10.8
Ireland	10	n.d.	n.d.	10		0.9
Italy	480	80	n.d.	560	1.0	7.0
Hungary	490	10	n.d.	500	0.2	10.9
Portugal	365	45	8	418	2.3	21.1
Russia	15,000	500	n.d.	15,500	0.4	12.6
Slovak Republic	134	37	7	179	2.6	12.6
Spain	1,250	600	550	2,400	4.5	18.0
Switzerland	80	12	10	102	2.9	25.4
United Kingdom	2,500	180	n.d.	2,680	3.1	45.6

Table 5 Extent of no-tillage adoption worldwide (Source: Derpsch, 2005)

Country	No-till (1,000 ha)
USA	25,300
Brazil	23,600
Argentina	18,270
Canada	12,520
Australia	9,000
Paraguay	1,700
Indo-Gangetic Plains	1,900
Bolivia	550
South Africa	300
Venezuela	300
Uruguay	260
Chile	120
Colombia	100
China	100

disease management are also obstacles to the diffusion of conservation agriculture. For instance, in recent years the increased incidence of head blight disease (*Fusarium* spp.) recorded in wheat and maize has been observed in no-till or minimum-till systems. However, choosing the least susceptible wheat variety along with fine chopping of maize residues leads to a lower head blight incidence (Vogelgsang et al., 2004).

Although it is reported that from the total area of the world under CA, only a small proportion is generally practiced on small farms (Wall and Ekboir, 2002) due to the limited investment made in research and technologies for small farmers. Nevertheless, in several parts of the world, adoption of no-tillage systems by small farmers has been increasing significantly in recent years. According to Brazilian national Zero-tillage Federation (FEBRAPDP) (Lutecia Canalli, 2005, "personal communication"), there are about 500,000 to 600,000 ha of no-tillage farming being adopted by small farmers with animal traction in Brazil. In Paraguay, the number of small farmers adopting the technology is growing rapidly. It is estimated that approximately 12,000 farmers are using no-till on about 30,000 ha. It is said that there are 300,000–350,000 small farmers with less than 1/2 ha adopting no-tillage systems in Ghana, but this information and the number of ha involved cannot be confirmed. Another region with a huge number of farmers adopting no-till on small farms is the Indo-Gangetic-Plains. Here, a few hundred thousand farmers are using the technology on an estimated 1.9–2.1 million ha. Regardless, significant effort will be needed to foster the adoption of CA in low-resource areas. This has the potential to provide large, long-term positive environmental effects, but it will require long-term investment in research, development, demonstration, and information networks. It is also crucial to have the farmers involved with experiments and demonstration in their own fields to accelerate the adoption of conservation agriculture (Derpsch, 2008).

5 Conclusion

Agriculture in the next decade will have to produce more food on less land and purchased production inputs by making more efficient use of natural and applied resources but with minimal negative impact on the environment. There is considerable evidence that CA can provide a wide range of agro-environmental benefits. CA system has been shown to be simultaneously both theoretically and practically able:

- to reduce the use of fossil fuels, farm expenses, CO_2 emissions, soil erosion, soil water evaporation, nitrate levels in the soil profile, herbicide mobility, and persistence.
- to increase soil macroporosity and biopores, soil aggregate size and stability, soil water retention and water-holding capacity, SOM and N content in soil top layers, P and K stratification, enzymatic activity, earthworm population, and biodiversity.

In all countries and regions where CA systems have had significant rates of adoption, the changes in farm production methods from conventional have reversed the former trend of declining crop productivity in the medium-term period and led to an economically, ecologically, and socially sustainable form of cropping. Yield improvement can lag through the initial transition years, showing signs of increase only after few years. However, it has been claimed that in the CA, yields can match or exceed yields from plow-based conventional systems.

Nevertheless, some aspects have to be carefully noted. In many of the CA systems, weed control represents one of the more important issues, and there is a need to know the biology and ecology of all the weeds that are occurring and the knowledge of their mechanical and chemical control. Weed control should be more precise, with careful use of herbicides.

Managing cover crops in CA is completely different from the same in conventional plow-based systems. Indeed, to kill green manure cover crops and leave the plant residues on the soil surface can determine difficulties, particularly in sowing and early development of the succeeding crop, especially in high or low-residue production areas.

For CA to be successfully adopted, there needs to be a continuous development of appropriate equipment. Indeed, in choosing a no-till seeding machine or planter, many variables have to be considered, such as soil condition, crop species, and interrow crop distance. In some circumstances—i.e., favorable wet areas—negative incidents of specific pests occur, requiring more use of preventives (genotype choice, crop rotation, nutrient management) and more precise curative methods (timing of fungicide application).

The use of crop rotation in this system is much more important than in conventional. By permitting higher crop diversification, rotation has a crucial positive impact on weed, pest, and disease control, as well as on crop nutrient management.

To avoid failures, an adequate level of knowledge is required before going into CA, and all the aspects of the production system must be considered interconnected.

The barrier of mental change still remains the main obstacle to the diffusion of this new approach in agricultural practices. The lack of knowledge on how to begin is often a reason for failure. Indeed, farmers need to acquire the basic knowledge before attempting to try the technology on their individual farms, and plan the change well in advance. The administrative requirements imposed in the Common Agricultural Policy, and the support of the Soil Protection initiative, as well as an opportunity to help countries honor the commitments acquired by them in the Protocol of Kyoto, can make it a highly useful tool—not only for farmers, but for governments.

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Recurrent Mass Selection for Routine Improvement of Common Wheat: A Review

G.F. Marais and W.C. Botes

Abstract The pursuit of sustainable wheat production has significant economic, social, and environmental relevance. Yield levels and stability thereof are determined by continuously changing and fluctuating biotic and abiotic stresses. Achieving higher and more stable yields requires constant genetic improvement of numerous aspects of new wheat cultivars. Targeted traits may include wide adaptation, abiotic stress such as drought and salinity, tolerance, polygenic non-specific disease resistance, pyramided disease resistance, etc. Broadly defined breeding objectives such as these involve complex, polygenic, genetic mechanisms that pose formidable challenges to breeders. Fortunately, a diverse array of increasingly more sophisticated biotechnological tools is becoming available. Advances in understanding the mechanisms that determine sustainability traits, coupled with versatile and unambiguous genetic markers and generation acceleration methodologies, foster new opportunities for selection of such traits, yet breeding methodologies need to be adapted in parallel to fully capitalize on the new technology.

Recurrent selection applied to a self-pollinator provides for a powerful breeding tool. Continuous cross-hybridization maximizes heterogeneity and forges new linkage associations in genes. Subsequent inbreeding helps to weed out deleterious recessive genes, fixes desirable genes in the homozygous state, and allows for accurate progeny testing. In the past, the difficulty of randomly intercrossing large numbers of selected wheat plants has frustrated the application of the technique, however this problem has been solved through the use of genetic male sterility in conjunction with the hydroponic culture of tillers that are cut and pollinated at anthesis. Thus, it is possible to randomly intercross hundreds of selected genotypes to produce large F_1 populations (upwards of 50,000 seeds).

A recurrent wheat mass selection program is being conducted at Stellenbosch University with the purpose of developing and testing the methodology. A highly heterogeneous base population was established and is being managed

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as a medium-sized breeding program. The experience we gained allowed us to streamline its execution, and herewith we review current methodology and progress made. Recurrent mass selection proved a simple, yet highly effective, technique that has major advantages compared to conventional wheat breeding methods; among these are reduced operational costs, accelerated selection progress, maximization of crossover and genetic recombination potential, and its suitability for broad breeding strategies.

Keywords Breeding · Disease resistance · Genetic male sterility · Population improvement · *Triticum aestivum* L

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1 Introduction

Conventional wheat selection methods involve three distinct stages, starting with the making of planned crosses: normally single, triple, back, or double crosses (Stoskopf, 1999). The second phase involves development of inbred/homozygous lines through single plant selection in the early segregating generations (pedigree method); bulk propagation in the early generations followed by single plant and line selection in the later generations (bulk-population method); and random inbreeding (single seed descent method) or production of homozygotes through androgenesis or wide crosses (doubled haploid method). The final phase of progeny testing of

inbred/homozygous lines is the same for the four methods. Annual crosses are normally planned based on the performance of advanced lines in the previous season, thus giving a cyclic long-term nature to the methodologies. New variations may be introduced regularly, normally through a process of prebreeding that limits cotransfer of undesired chromatin and preserves earlier selection gain. Objections to these methods include limited opportunities for genetic recombination and difficulty balancing simultaneous stringent selection of simple traits with high heritability and complex traits with low heritability. Backcross breeding, whereby single, desirable traits are entered into a commercially proven genetic background, is often employed in a supplementary manner in conventional breeding. The purpose may be to upgrade an otherwise superior genotype lacking a critical trait; to provide for prebreeding during introgression of a new trait; or to develop multilines or near-isogenic lines. Major drawbacks of the backcross approach are that it may impose selection ceilings and, if not used with discretion, may seriously narrow the genetic variability of a breeding population.

In conventional breeding of self-pollinating crops, genes are rapidly fixed through self-fertilization following the initial cross, and heterozygosity is halved with every successive filial generation (Stoskopf, 1999). This strongly reduces the opportunity for genetic recombination. In dealing with polygenic traits, it is therefore totally unrealistic to expect that the polygenic recombination potential of a cross can be adequately explored in a single cycle of crossing and selection. Recurrent mass selection is a well-established breeding technique for the genetic improvement of cross-pollinating species (Hallauer, 1981). It was developed primarily for the improvement of quantitatively inherited traits (controlled by numerous genes, each with small effect and modified by the environment), and the underlying objective is to systematically increase the frequency of desirable genes in a breeding population so that opportunities to extract superior genotypes are maximized. The inherently high level of heterozygosity in the breeding population coupled with a large number of cross combinations allow for more complete exploration of polygenic recombination potential (Jensen, 1970; Hallauer, 1981).

The principles underlying recurrent mass selection are equally applicable to autogamous crops, but the difficulty associated with intercrossing in each cycle, coupled with small amounts of seed produced, has discouraged its use. This led Hallauer (1981) to suggest that recurrent selection procedures should preferably be integrated with other selection methods, and that its products could not normally be expected to be directly useful for commercial cultivar development. Pilot studies that applied recurrent selection to self-pollinating small grain cereals generally had positive outcomes; however, these studies were mostly short-term (less than five cycles), were restricted in terms of the number of intercrosses that could be made, and pursued single traits (Wiersma et al., 2001). Wiersma et al. (2001), Díaz-Lago et al. (2002), and Liu et al. (2007) provided excellent overviews, highlighting the effectiveness of recurrent selection for genetic improvement of grain protein, kernel weight, grain yield, or disease resistance in numerous experiments with crops such as soybean, barley, wheat, and oat. Impressive selection progress was recorded for complex traits that are difficult to breed by

conventional means—for example, grain yield of oat (De Koeyer and Stuthman, 1998), groat oil content of oat (Frey and Holland 1999), and partial resistance to oat crown rust (Díaz-Lago et al., 2002). In China, recurrent selection based on the Taigu source of male sterility was used to pyramid minor and major genes for scab resistance, improved salt tolerance, drought tolerance, and yield potential (He et al., 2001). However, the focus on single traits often resulted in undesirable correlated changes in unselected traits, thus reducing the usefulness of the strategy and highlighting the necessity for a holistic approach to its use in cultivar improvement (Wiersma et al., 2001).

Wallace and Yan (1998) stressed the importance of a systemic rather than focused approach to plant improvement. The living plant is a complex biological system driven and regulated by extensive and interdependent genetic, epigenetic, and environmental mechanisms. Photosynthate is apportioned for biomass accumulation and harvestable product in accordance with availability and the requirement of metabolic pathways, plant defense, and stress tolerance mechanisms. Selection for a specific trait is therefore likely to impact on interconnected pathways (Comeau et al., 2007), thus affecting overall plant performance. A well-adapted genotype appears to be better able to optimize its responses to the physiological demands of a particular environment, and to breed and select these genotypes, breeding should be less focused on narrowly defined objectives and should be more holistic and multidisciplinary based (Wallace and Yan, 1998; Comeau et al., 2007). During selection, a broad range of biotic and abiotic stresses relevant to the targeted production region should be applied. When done in conjunction with recurrent or convergent breeding strategies, this could aid the development of broadly adapted genotypes.

Jensen (1970) suggested the diallel selective mating system as a means to accommodate recurrent selection in self-pollinating species that are difficult to cross, and that produce few seeds per cross. This strategy integrates recurrent selection principles with conventional breeding strategies, and thus allows for simultaneous genetic input of a broader range of parents, breaking up linkage blocks, and freeing genetic variability by fostering genetic recombination. Jensen (1970) furthermore suggested the use of male sterility to facilitate crossing, and pointed out that the breeding material should be selected in specialized environments to maximize the genotypic expression of the traits being selected. McProud (1979) pointed out that the majority of established cereal-breeding programs around the world have a cyclic nature and may contain an element of recurrent selection. Such programs involve the generation of variability through crosses, followed by the derivation and evaluation of inbred lines. Superior lines are then intercrossed to sustain a next cycle of selection. While they have been successful, these programs are hampered by long selection cycles, and breeders need to guard against genetic bases that are too narrow and not enough introgressions of new variability. To maintain selection progress, it is necessary to introgress new useful variation in a controlled manner that would minimize the introduction of deleterious genes and preserve existing favorable gene combinations. Falk (2002) developed and tested a recurrent selection strategy called recurrent introgressive population enrichment (RIPE) in barley that was designed to address the above shortcomings of conventional breeding methodologies. The

system is based on a recessive gene for male sterility (*msg6*) closely linked in a coupling phase with a recessive, xenia-expressing shrunken endosperm gene (*sex1*). F_2 seeds with shrunken endosperm primarily develop into male-sterile (female) plants. Crosses and F_1 multiplication are done in growth rooms during the off-season, the F_2 is planted in the field in May of Year One; the F_3 is grown in an off-season nursery; and the F_4 is evaluated in an unreplicated field trial. Selected lines provide male parents in the next cycle of crosses and are also destined for advanced testing in multiple-location trials. Crosses are made annually and thus the duration of the female selection cycle (one year) differs from the male selection cycle, which extends over two years. A limited number of crosses (NC Design 1) are made each year, but the elite population is continuously enriched with new variation; this is achieved through eight generations (three years) of crosses with the elite population, resulting in the introgression of approximately 6% new alleles at a time. World Wide Wheat (W^3) is a wheat breeding company that commercially employs male sterile facilitated recurrent selection (MSFRS). Compared to conventional pedigree breeding, the recurrent methodology claims to allow for more rapid and efficient cultivar development (http://www.worldwheat.com/company_overview.php).

The base population under recurrent selection should be assembled carefully to contain adequate genetic variability. It should provide scope for the genetic improvement of adaptation, production, and processing characteristics peculiar to the crop and targeted production region. A recurrent selection program aimed at increasing kernel weight in spring wheat was initiated at North Dakota State University in 1967 (Busch and Kofoid, 1982). Following a screening of about 100 cultivars and breeding lines for kernel weight, the ten best lines were intermated in the 45 possible cross combinations to form a base population. Subsequent cycles were generated through manual crosses of representative numbers of selected plants from the segregating generations.

Making use of the dominant male sterility gene, *Ms2* (Taigu source), Huang and Deng (1988) established a recurrent selection breeding population segregating for male sterile (female) and male fertile plants. In their system, selected female plants were naturally (field) pollinated by selected male fertile plants. A small, nation-wide network of Chinese researchers pursued recurrent selection-based applications, and released a number of new cultivars (He et al. 2001). Cox et al. (1991) developed and registered a germplasm source segregating for the presence of the dominant male sterility gene, *Ms3* (derived by Maan and Williams 1984). Over the course of several years, numerous sources of diverse disease- and insect-resistant, quality and yield genes were involved as parents to end up with a highly heterogeneous base population. Cross-pollination of male sterile spikes by fertile plants (greenhouse) was enhanced using fans and manual agitation. Marais et al. (2000), made use of the dominant male sterility gene *Ms3*, to establish a recurrent selection base population consisting of 50% male sterile plants. They developed a simple hydroponic system to achieve large-scale random intercrossing of the selected plants in a greenhouse. Liu et al. (2007) established a recurrent selection base population by pollinating a source with the D^2-type of cytoplasmic male sterility, with 30 diverse elite cultivars and lines derived from six provinces in China.

Male sterility genes provide an easy means to obtain female plants for use in recurrent selection applications. However, numerous chemical hybridizing agents have been described (Chakraborty and Devakumar, 2006) that provide an alternative to the use of genetic or cytoplasmic male sterility for the implementation of a hybridization strategy.

The overall goal of recurrent selection is to increase the frequency of desirable genes in the base population, yet maintain genetic diversity while doing so (Wiersma et al. 2001). Continued selection progress depends on the amount of variation present, heritability of the trait, and the initial frequencies of desirable alleles. Past studies have suggested that for complex, quantitatively inherited traits, steady selection progress can be maintained for many cycles. If properly managed to avoid sampling effects, variation for unselected traits can similarly be maintained within the recurrent population (De Koeyer and Stuthman, 1998; Frey and Holland, 1999; Wiersma et al., 2001). It is also necessary to provide for continuous introgression of new, useful genes in the base population. This needs to be done in a manner that will not undo the selection progress already achieved, and therefore has to involve backcrosses to the base population (Marais et al., 2001a; Falk, 2002).

2 Sustainability of Rust Resistance

Commercial wheat production relies on genetically uniform, high-yielding cultivars that are grown over large areas of land, often for many years in succession. Resistance to the rusts is often based on single, major genes that exert strong selection pressure on the pathogen and, as a consequence, may be short-lived. “Arms races” result between wheat breeders and the pathogen that necessitate an ongoing search for new, effective rust resistant genes to employ and steer pathogen evolution in specific directions (Knott, 1989). This situation contrasts starkly with the high level of genetic diversity in natural grass populations, land races, and crop mixtures that historically serve to buffer the spread and evolution of a pathogen.

The desirability of reintroducing genotypic diversity of resistance in modern-day commercial wheat production has been advocated (Browning, 1988; Knott, 1989). Suggested ways in which this can be achieved include the use of species mixtures, cultivar mixtures, and clean and dirty multiple lines. A cultivar mixture is a comparatively simple strategy that effectively reduces the damage caused by rust diseases (Browning, 1988; Wolfe, 1988) but could compromise uniformity (Groenewegen and Zadoks 1979). Interfield diversity and regional deployment strategies to slow down pathogen progression are difficult to manage and are often not widely accepted by farmers (Frey et al., 1979; Knott, 1989). Multiple lines were developed in an attempt to improve the agronomic and technological disadvantages of cultivar mixtures (Groenewegen and Zadoks, 1979). The development of the near-isogenic or phenotypically similar components and continued maintenance of multiple lines are time- and effort-consuming, and the strategy is limiting as far as genetic progress with selection for nondisease traits is concerned (Frey et al., 1979; Groenewegen and Zadoks, 1979). However, multiple lines are reportedly effective

in slowing down disease progression (Browning et al., 1979; Browning, 1988; Frey et al., 1979).

In cross-pollinators, recurrent selection can be used to develop and continuously improve open-pollinated cultivars. If the same principle is applied to a self pollinator, it is possible to breed cultivars that would be akin to modern land races. In such an approach, numerous diverse resistance genes may be introduced in the recurrent base population and selected to establish different gene frequencies depending on the nature of the pathogen population. The base population that is released as a "land-race" cultivar can be diversified continuously in terms of its resistance, and at the same time be selected for uniformity (agrotype and processing quality) and improved agronomic performance. To counter shifts in the resistance gene frequency that are due to natural selection, new foundation seed can regularly be derived from the base population. Compared to a multiple line, and as a result of the high level of heterogeneity and recombination in the base population, a "land-race" cultivar could include a much wider array of genotypic combinations of resistance genes that also exploit complementation, interaction, and additive effects of genes.

An alternative to intracultivar genotypic diversity is to pyramid universally effective resistance genes in a single genotype. This can also provide a more durable barrier to pathogen adaptation, which requires the pathogen to simultaneously mutate at a number of loci to be able to overcome the complex, polygenic resistance (Knott, 1989). Both the multiple line and gene pyramid strategies require a thorough knowledge of the dynamics of the pathogen population, and continuous adjustment with respect to genes that become ineffective and are complicated by the simultaneous use of the same genes in cultivars with single gene resistance (Browning 1988; Wolfe, 1988; Knott, 1989). Pyramiding of major resistance genes is complicated by the fact that the gene with the strongest effect masks the presence of genes with lesser phenotypic expression, and is thus best achieved with the use of markers, allowing all genes present to be known. Most attempts to pyramid resistance are based on backcrosses or convergent crosses that impose a yield ceiling and make it problematic to add onto existing gene pyramids. Recurrent selection, on the other hand, allows for continued pyramiding, without the sacrifice of selection progress for other traits (Pretorius et al., 2007).

The pursuit of durable resistance may be a more sensible approach towards achieving sustainable rust resistance. Polygenic, nonspecific resistance is conditioned by the presence of a number of genes, each with small effect on the total resistance phenotype. Should virulence develop for one of the components, this should result in only a small phenotypic effect. Polygenic, nonspecific resistance is postulated to be incomplete (yet this need not always be the case) and to exert only mild selection pressure on the pathogen (Parlevliet, 1988; Knott, 1989). Past experience has shown that partial or durable resistance is not necessarily polygenic (McIntosh, 1992; Rubiales and Niks, 1995). Similarly, while adult plant resistance is often partial (Bariana and McIntosh 1995) and likely to be durable (McIntosh, 1992), it can either be race-specific or race nonspecific (Kaur et al., 2000).

By definition, durable resistance remains effective after widespread use over a long period of time (Johnson and Law, 1975). Since durability is difficult to measure,

it is problematic to breed for and generally entails selection for component traits. Breeding for durable, polygenic resistance requires the use of a pathotype that is virulent on all the parents in the seedling stage, or the prior elimination of cross progenies with genes for specific resistance. Selection then needs to focus on aspects such as incubation period, and number and size of uredia (Browning, 1988; Parlevliet, 1988; Knott, 1989). In areas where more than one rust disease is of importance, attempts to develop durable resistance may need to simultaneously address all, which may be difficult (Groenewegen and Zadoks, 1979; Frey et al., 1979).

Parlevliet and van Ommeren (1988) demonstrated that mild recurrent mass selection against susceptibility provided a powerful tool for the accumulation of partial resistance genes (in the absence of major race-specific genes and employing a defined pathogen population). The shadowing effect of race-specific resistance genes on phenotypic selection for durable resistance limits its integration in commercial breeding. This can be overcome by prior genetic analysis of appropriate segregating populations to identify and tag quantitative trait loci (QTL) for durable resistance that would allow marker-assisted breeding of the trait (Castro et al., 2003; Balakrishna et al., 2004).

Ultimately, the most appropriate strategy for achieving sustainable genetic control of rust diseases would be to combine single or multiple genes for durable resistance with major race-specific genes. This can be achieved by pyramiding the target genes in a single genotype or by combining genotypes with diverse resistances in a multiple line or a land-race-type cultivar. Such strategies will be technically challenging and will, of necessity, have to rely strongly on the availability of tightly linked molecular markers. While back- or convergent-crossing schemes are often employed in gene pyramiding and for the development of multiple lines, they impose yield ceilings. Recurrent selection, on the other hand, provides a highly effective alternative procedure of gene pyramiding and breeding for land-race cultivars that does not limit the genetic improvement of any other trait. Furthermore, recurrent selection is the breeding scheme best suited to holistic breeding objectives that encompass rust resistance, broader disease, and pest resistance, wide adaptation, yield, and quality. Because it is a population improvement strategy, it enables a breeder to focus on specific breeding targets at a given time without forfeiting the opportunity to subsequently select for other traits. Provided that the population is large enough and selection bottlenecks are avoided, variation for unselected and uncorrelated traits should remain unaffected, allowing for continued and intense recombination and exploitation.

3 Establishing a Recurrent Mass Selection Base Population at Stellenbosch

A genetically diverse base population, rich in genes for adaptation, quality, yield, and pest resistance, and segregating for male sterility was established. Accession KS87UP9 (Cox et al. 1991) of winter wheat segregating for the dominant male

sterility gene, *Ms3* (Maan and Williams, 1984), was obtained from the USDA-ARS, Dept of Agronomy at Kansas State University. A male sterile KS87UP9 plant was pollinated with the spring wheat, "Inia 66," and sterile F_1 plants were pollinated with a spring wheat breeding line. Male sterile F_1 with a spring growth habit were then used in a multicross with seven spring wheats. Sterile male multicross F_1 plants were subsequently randomly intercrossed with 60 wheat breeding lines with diverse disease resistance. In the following cycle (1999), 44 selections from a pedigree program and 157 selections from the recurrent program were used as male population. In 2000, the male population consisted of 64 selections from the pedigree breeding program and 157 selections from the recurrent population. In 2001–2004, the recurrent F_1 was annually crossed with 60–120 selections from a pedigree breeding program. As will be shown in later sections, the selection cycle in this program extends over four years, and in each year the F_1 of the previous year was used as a female parent. As a result, the genetic contributions of the various parental populations to the base population could be estimated as: (a) 2000 F_1 female population : 12.5%; (b) 2001 male population : 12.5%; (c) 2002 male population : 23%; (d) 2003 male population : 24%; and (e) 2004 male population : 30%. In 2005, the base population was closed and male plants were only selected from within the recurrent mass selection base population. Subsequently, new variations would only be added once it had gone through a cycle of "recurrent backcrossing."

3.1 The Recurrent Mass Selection Breeding Plan

Marais et al. (2001a, b) proposed a recurrent mass selection strategy for wheat that is outlined in Fig. 1 . The duration of a breeding cycle is shown to be four years whereas the male and female components are handled differently. In this scheme, male selection (F_6) is based on performance in an unreplicated single row at a single locality. This allows for a four-year breeding cycle that is an advantage in the initial stages of selection in a highly heterogeneous base population. Strict initial selection for simple, highly heritable traits (while maintaining large populations) means that the base population can be enriched for these traits without loss of heterogeneity for complex traits of low heritability. However, in the longer run, the breeding cycle may be extended to allow for better sampling of the target mega environment and to improve selection gain for quantitative traits of low heritability.

3.2 Making of Crosses and Hydroponic Maintenance of Cut Tillers

To facilitate intercrossing, male sterile and male fertile spikes of selected plants are cut at the time of flowering and kept in hydroponic solution to effect pollination, where only the female tillers are maintained until the seeds ripen (Fig. 2). Galvanized iron trays with dimensions of 600 mm x 450 mm x 160 mm (and coated on the inside with black antifungal paint) are used that can each accommodate 230

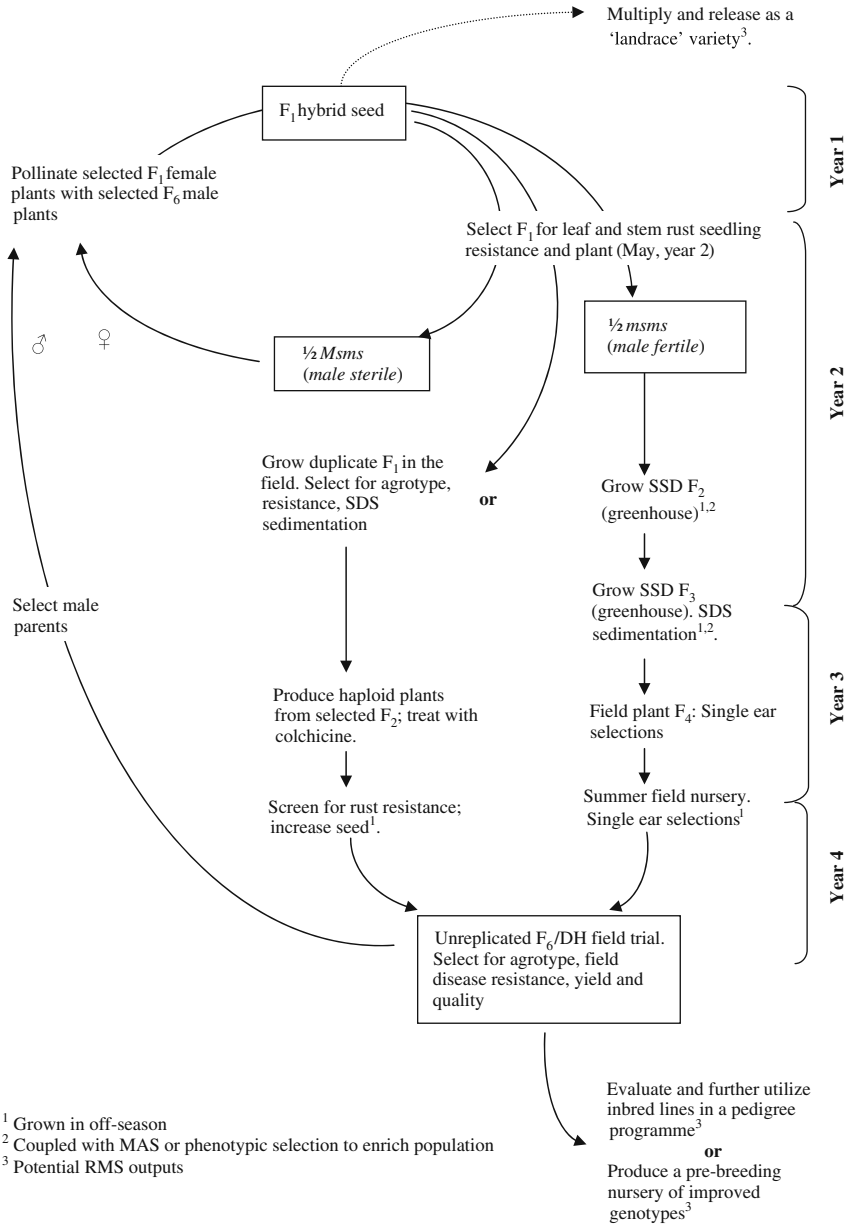
Multiply and release as a 'landrace' variety[3].
F1 hybrid seed
Pollinate selected F1 female plants with selected F6 male plants
Select F1 for leaf and stem rust seedling resistance and plant (May, year 2)
Year 1
Year 2
Year 3
Year 4
♂
♀
½ Msms (male sterile)
½ msms (male fertile)
Grow duplicate F1 in the field. Select for agrotype, resistance, SDS sedimentation
or
Grow SSD F2 (greenhouse)[1,2]
Grow SSD F3 (greenhouse). SDS sedimentation[1,2].
Select male parents
Produce haploid plants from selected F2; treat with colchicine.
Field plant F4: Single ear selections
Screen for rust resistance; increase seed[1].
Summer field nursery. Single ear selections[1]
Unreplicated F6/DH field trial. Select for agrotype, field disease resistance, yield and quality
Evaluate and further utilize inbred lines in a pedigree programme[3]
or
Produce a pre-breeding nursery of improved genotypes[3]
[1] Grown in off-season
[2] Coupled with MAS or phenotypic selection to enrich population
[3] Potential RMS outputs

Fig. 1 Common wheat recurrent mass selection scheme (abbreviations: DH = doubled haploid; MAS = marker-assisted selection; RMS = recurrent mass selection; SSD = single-seed descent)

Fig 2 Making of crosses: (**a**) F_1 population segregating for male sterility; (**b**) male sterile (female = *Msms*) spike; (**c**) random female spikes being pollinated with random male spikes; and (**d**) hybrid seeds ripening on the female tillers

female spikes. Up to 70 male spikes are placed into two narrower trays positioned on either side of the main tray and raised about 600 mm. The main tray is fitted with inlet and outlet holes for changing the nutrient solution without having to remove the female spikes. Enough spikes to fill 1–2 containers are collected twice a week over a period of six weeks. This results in a total of 12–15 trays (approximately 2,760–3,450 female spikes). Each container is filled with 40% nutrient solution and 0.01% "Jik" (household detergent containing 3.5% sodium hypochlorite; Reckitt and Colman South Africa Pty Ltd, Elandsfontein, South Africa) in tap water. The nutrient solution (pH neutral with electrical conductivity = 1.5–2.0 dSm^{-1}) is made up of 164 g Sol-u-fert T3T (Kynoch Fertilizers Pty Ltd, Milnerton, South Africa), 2 g Microplex (Ocean Agriculture Pty Ltd, Muldersdrift, South Africa) and 77 ml

potassium nitrate in 100 L H_2O. The containers are kept in a growth chamber with 14 h day/10 h night cycles at 16°C/12°C for the duration of grain filling. An air pump is used to continuously aerate the solution. Female tillers are cut just below the second-to-last internode, and care is taken to keep the flag leaf intact. Florets on female tillers are cut open to facilitate pollination and, in the process, incompletely male sterile spikes are discarded. Male tillers are collected in buckets, stripped of their leaves, and arranged above the female tillers. As a result of the extensive handling, male tillers shed all their ripe pollen by the time they are arranged in the containers. This means that the next pollen shed is more or less synchronized among tillers. After allowing 5–6 days for pollination, the male tillers are discarded. Female tillers are trimmed and transferred to fresh nutrient solution once every two to three weeks.

3.3 The Crossing Block

Each year, 9,000–12,000 F_1 hybrid seeds from the preceding season are planted in a growth chamber and seedlings are screened with an inoculum mix consisting of 5–8 *Puccinia triticina* (leaf rust) and 3–5 *P. graminis f. sp. tritici* (stem rust) pathotypes (Fig. 1). Approximately 3,000 seedlings exhibiting the lowest levels of infection are transplanted to the crossing block. Fifty percent of these will be male sterile plants and can be used as females (the remaining 50% of male plants are used to initiate single-seed descent inbreeding). F_6 inbred lines (100–120) that were field-selected in the previous season and originate from crosses made four years earlier are used as the male parents. In our system, seed set is about 90–95%, and we produce roughly 60,000–70,000, hybrid seeds per season. This is about five times the amount of seed we manage to screen. The 1,000-grain mass of seeds is about 16 g. While small, the seeds are well-developed and result in 85–90% germination.

3.4 The Breeding Cycle

Selection is done on both the male sterile and male fertile components. However, only the male fertile populations are field-tested. The male fertile plants are advanced rapidly from the F_1 to the F_5. Following the evaluation of F_6 rows (unreplicated trial) for yield, agrotype, disease resistance (supplemented with marker-aided selection), and quality, the superior selections are used as male parents for hybridization. In conformance with funding conditions (Winter Cereal Trust), a set of lines with commercial potential is also identified at this time and distributed as an annual nursery to local wheat breeders (thus, the project also serves as a national prebreeding program). Had it not been the case, advanced progeny testing and selection of the F_5-derived lines would have been done the same way, as in a regular breeding program for a self-fertilizing crop.

The F_1 male plants are advanced to the F_4 before field planting in May of the ensuing year (Fig. 1); growing two single seed descent populations in an uncooled greenhouse during the summer makes this possible. The F_5 is grown under irrigation in the summer to yield F_6 seed for unreplicated trials in May of the third year. Thus, generation acceleration comparable to doubled haploid technology is achieved through single-seed descent and summer planting. Advantages compared to doubled haploids are that it is much cheaper and larger numbers can be handled. Furthermore, it is possible to do seedling leaf and stem rust resistance and quality (sodium dodecyl sulphate (SDS) sedimentation) screens and selection at the start of each single-seed descent cycle. As a result, a large proportion of lines that are deficient in these respects can be discarded early on, making the process more cost-effective. Population size need not be compromised by strict selection, as larger numbers may simply be involved in the initial single-seed descent phases. Single-seed descent makes it possible to limit the total selection cycle to four years, which is a huge advantage in terms of realizable selection gain. The expected duration from the time a cross is made to the time a cultivar is released can be reduced accordingly (from 12–13 years to 7–8 years) and is similar to the length of the breeding cycle in doubled haploid breeding.

In a warm climate such as the Republic of South Africa, generation acceleration is ideally achieved through single-seed descent inbreeding. Short growing season cultivars are being bred, and by providing supplementary light during late fall, we manage to produce three generations per year. However, if resources are not limiting, or when dealing with intermediate or winter wheat, it would be preferable to produce doubled haploids instead. This alternative is also shown in Fig. 1. A general problem associated with the development of doubled haploids from heterogenous genotypes is the high number of lines that are subsequently discarded on the basis of highly heritable, simple traits, such as disease susceptibility (Kuchel et al., 2005). It would therefore be sensible to grow the male fertile F_1 as a space-planted population in the field, and to select single plants on the grounds of agronomic and disease phenotype to be used for doubled haploid production in the third year. F_2 segregates may again be seedling screened in a greenhouse before use. In the fourth year, a field-planted doubled haploid nursery can be grown and screened for agronomic, disease, and quality attributes. This population will then serve as a basis for the selection of male parents and genotypes destined for advanced testing. Whereas use of doubled haploids in recurrent selection may initially be complicated by genotypic variation for wide crossability or androgenetic response (Eudes and Amundsen, 2005), the frequency of genes that promote haploid production should increase over time in the recurrent mass selection population as a result of its indirect selection, thus facilitating doubled haploid production.

To facilitate selection for broad adaptation, it is possible to grow each of the F_4 and F_6 populations (single-seed descent option; Fig. 1) at different localities that will exert different environmental pressures. It is even possible to involve up to four localities in the course of a four-year cycle. Over time, the recurrent population is expected to become enriched with favorable alleles and it may then be advantageous

to increase the selection cycle to five or six years, thereby creating an opportunity for more precise multiple locality testing of inbred/doubled haploid lines to improve the selection of quantitative traits.

3.5 Effective Population Size

Since the selection cycle extends over four years, the effective size of the base population is determined by the material handled within a four-year period. Involvement of comparatively large numbers of genotypes further buffers the effect that very strict selection (small groups of selected male parents) may have on the base population's genetic background. Since the recurrent mass selection system can easily produce large numbers of progeny, it is advisable that very strict selection should always be offset by an increase in the size of the selected population.

4 Raising Allele Frequencies through Recurrent Mass Selection

Recurrent selection may be very effective in raising the frequency of single major genes of high heritability, such as those for wheat leaf-, stem-, and stripe rust resistance, as well as genes for which highly diagnostic markers are available. It will be equally effective to reduce the frequency of an undesirable gene. Figure 3 (a) shows the effect on allele frequency when the F_1 only, the F_6 only, or both the F_1 and the F_6 are selected (making use of the selection scheme outlined in Fig. 1 and with the single-seed descent option), assuming dominance and an initial allele frequency of 5%. It is evident from the graph that with selection in the F_1, allele frequency changes gradually during four cycles and then increases sharply when the effect of selection is also being carried through the male parent. F_6 selection (F_5-derived inbred lines) is far more efficient in raising gene frequencies. One and two cycles of F_6 selection is predicted to raise gene frequencies to higher levels than five and ten cycles of F_1 selection, respectively. If the male population is inbred for five generations, resistant plants selected in the F_6 are mostly homozygotes, primarily contributing gametes with the desired allele to the next generation (the process is therefore comparable to a backcross). Combined F_1 and F_6 selection is even more effective, but would be relatively costly.

Clearly, strict selection of the male parent (F_6 inbred lines) should only be done when gene frequencies are sufficiently high to prevent bottlenecks, or if large populations can be screened to ensure maintenance of genetic diversity for other characteristics. Should the initial frequency of an allele be very low, phenotypic or marker-assisted selection can be done at the onset of single-seed descent inbreeding to enrich the F_6 field population in terms of the targeted genotypes, thus avoiding loss of heterogeneity through the use of small male populations.

If a base population is being selected with the purpose of deriving single genotypes with pyramided resistance, it may suffice to simply raise the frequencies of desirable resistance alleles to levels higher than 0.70. If the base population is being developed into a land race cultivar, it may be necessary to fix the frequencies of

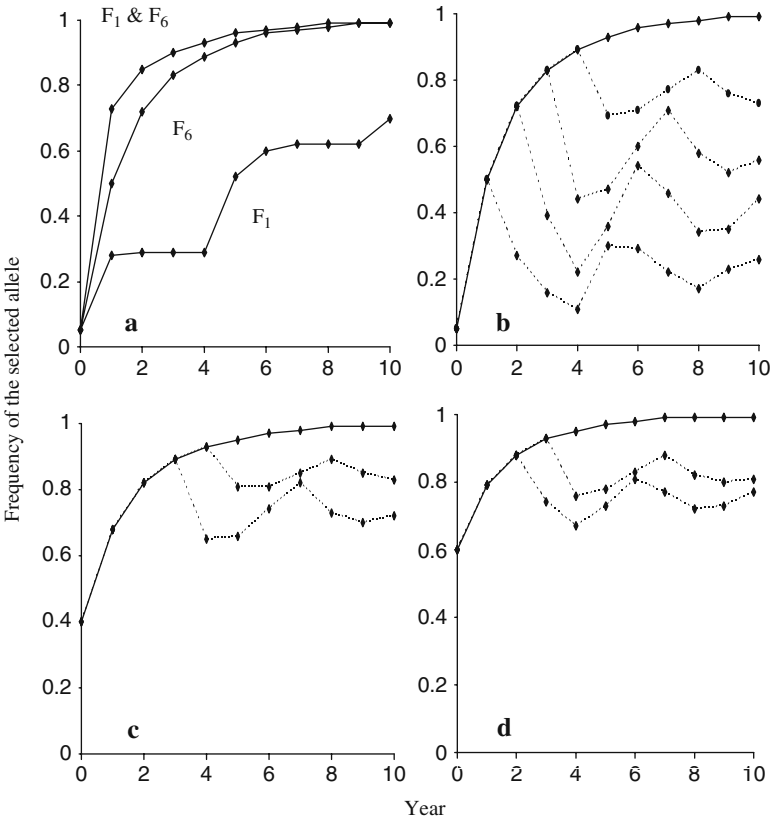

Fig. 3 (**a**) Expected change in the frequency of a target allele (initial frequency, p = 0.05) if selection is done in the F_1 only; in the F_6 only; or in both the F_1 and the F_6. (**b**), (**c**) & (**d**) Expected changes in target allele frequencies when selection is done in the F_6 only. Also shown (broken lines) are the subsequent fluctuations in gene frequencies when selection is stopped after (**b**) one, two, three, or four years (initial p = 0.05); (**c**) three or four years (initial p = 0.40); and (**d**) two or three years (initial p = 0.60)

genes affecting phenotypic uniformity, and to raise the frequencies of resistance genes in accordance with the frequencies of virulence genes in the pathogen population (Frey et al., 1979). Figure 3b, c, d shows the effect of terminating selection (F_6) at different stages for three different initial gene frequencies. If the frequency of the desired allele is initially very low, say 5% (Fig. 3b), then at least four seasons of selection are required to raise it to a level higher than 70%. When the initial frequency is about 0.40 (Fig. 3c), three seasons of selection will raise it to an average level exceeding 70%. When the initial frequency is 0.60 (Fig. 3d), two seasons of selection will raise it in excess of 70%.

5 Marker-Assisted Breeding

As gene marker technology continues to improve, it finds ever more application in routine breeding; however, compared to phenotypic measurement, the use of markers remains costly. It is therefore critically important to determine at which stage of a breeding program markers should be employed to ensure maximum genetic effectiveness and economic efficiency (Bonnett et al., 2003; Kuchel et al., 2005). In the present recurrent mass selection program, markers can be employed for F_1 screening; for screening during single-seed descent inbreeding, or for screening of the F_5-derived F_6 inbred lines for use as parents. From Fig. 3, it appears that marker-assisted selection in the F_6 would genetically and cost-wise be the most effective strategy. Marker selection done during single-seed descent inbreeding cannot be more effective than F_6 selection in raising the frequency of the target allele, and of necessity will involve a higher volume of analyses.

Due to the high level of heterogeneity in the base population, markers should ideally be allele-specific. Such markers are normally easy to obtain for genes carried on alien translocation segments, yet are scarce in the case of genes that are of common wheat ancestry. However, if closely linked markers are available for a gene that is of common wheat origin, it may still be used effectively if a high level of linkage disequilibrium exists within the population. In this case, it will be necessary to determine the frequencies of the possible marker-trait associations that exist within the breeding population beforehand, making use of a random sample of genotypes. If a marker is positively linked to the target trait in a sufficiently high proportion of the population, it can still be used to raise frequencies to desired levels.

5.1 Use of Markers in Conjunction with Recurrent Mass Selection to Pyramid Rust Resistance Genes

In the recurrent mass selection scheme of Fig. 1 (single-seed descent option), the percentage of F_6 inbred lines that can be expected to carry the target alleles for various numbers of (independent) genes and gene frequencies were calculated by Marais and Botes (2003). For example, when the frequencies of the target alleles are 0.7 with 10 genes targeted, about 2.6% of the inbred lines will carry all ten genes (in hetero- or homozygous form). With 15 genes targeted (allele frequencies 0.7), about 0.4% of the F_6 can be expected to carry all of them. If the frequency of the desired allele at each of 20 loci is 0.80, about 1% of the inbred plants are expected to contain them all. Numerous other inbred lines will clearly have diverse combinations of fewer target genes that may still be very useful. Thus, if carefully managed, the recurrent population will in time become a sustainable and continuously improving source of diverse pyramided genotypes.

5.2 Evaluation of F_6 Inbred Lines

About 1,500 new F_6 inbred lines annually are field-tested in an unreplicated trial. Each line is planted as a single 3 m row, and is compared against the most successful commercial cultivars. Disease spreader rows are used to ensure that epidemic conditions will develop. Apart from natural infection, the spreaders are inoculated with prevalent pathotypes of *Puccinia triticina* (leaf rust), *P. graminis f. sp. tritici* (stem rust) and *P. striiformis f. sp. tritici* (stripe rust). Each F_6 line is scored for *Blumeria graminis f. sp. tritici* (powdery mildew), leaf rust, stripe rust, stem rust, and *Stagonospora nodorum* (Septoria leaf and glume blotch) reaction, growth habit, plant type, straw strength, shattering, yield, quadrumat extraction, and mixograph characteristics. In addition, DNA extracts are made and lines screened by polymerase chain reaction (PCR) for the presence of targeted resistance genes. Marker-assisted selection is most cost-effective if done following the F_6 field and quality evaluation of inbred lines. For this purpose, F_7 seeds ($\pm$ 4–5 seeds per inbred line) of selected lines are germinated in plastic dishes (25 compartments). The 1–2 mm long coleoptiles are harvested and pooled for DNA extraction.

Before the male parents are selected, it is possible to confirm that a targeted gene does not have negatively associated effects, and to abort its selection if it does. To do this, the total data set can be analyzed to confirm that there are no undesirable associations. Data contrasts may be derived to test whether the presence of the target gene may result in deleterious agronomic effects such as may be the case with some species-derived translocations. Following evaluation, the best 110–120 lines are selected to be used as male parents. Since 2006, the best material is also included in a nursery of prebred resistant genotypes distributed to local breeders who will evaluate it for direct release or use as parental material. The 2006 and 2007 nurseries were well received by breeders and were primarily used in crosses to improve the levels of rust resistance in their breeding populations.

5.3 Introgression of New Resistance Genes through Recurrent Backcrosses

To sustain genetic improvement, it is necessary to continuously introgress new and useful genes. To prevent degradation of the base population by the introduction of poorly adapted germplasm, new genes can be integrated via a series of backcrosses to the recurrent mass selection population. Currently, the genes *Lr19*-149 (lacking *Sr25* as well as the yellow pigment genes; Marais et al., 2001b), *Lr21*, and the *Sr31* complex ($1BS_{38:9}$.1BL translocation that lacks *Sec-1* yet has the *Gli-1/Glu-3* loci; Lukaszewski, 2000) are being introgressed through recurrent backcrosses.

The germplasm carrying the targeted genes is used to pollinate male sterile spikes taken from the most recent F_1 of the base population (Fig. 4). F_1 carrying the target genes are then backcrossed to the most recent F_1 base population. Such backcrosses need to involve large numbers of plants, particularly the final backcross. It appears

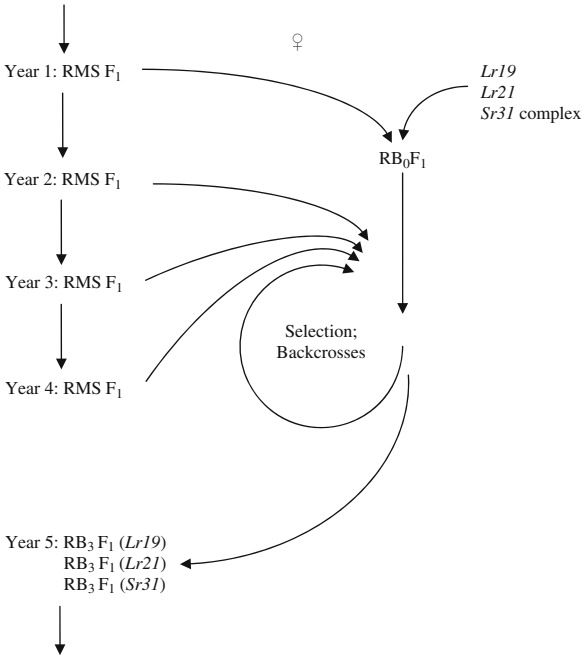

Fig. 4 Recurrent backcross strategy used to deliver new resistant genes to the recurrent mass selection base population (Abbreviations: RB = recurrent backcross; RMS = recurrent mass selection)

that three backcrosses are adequate. Following the third backcross, the recurrent backcross population retains an average of 6.25% of the donor germplasm, and is substituted for the normal recurrent mass selection F_1 population. The first male parents carrying the newly introgressed genes will be developed from this substituted population, and these will therefore only be available for crosses four years later. Due to this dela, the average percentage of foreign chromatin (in the F_1) will continue to be diluted for another three years and then will raise slightly as the first male parents with introgressed genes come into use. The percentage of foreign chromatin introduced should eventually stabilize at between 2–2.5%.

Recurrent backcrosses may also be used as an alternative to prevent bottlenecks arising from the initial selection of a gene that occurs at very low frequency in the base population. By initiating a subpopulation and continuously backcrossing to the latest F_1 while involving large enough numbers, the frequency of the gene can be raised without skewing or loss of background diversity.

6 Conclusion

If properly managed, recurrent mass selection is very effective. In our program molecular marker-assisted selection for rust resistance was not introduced until 2005. Prior to that, selection was based on the resistance phenotype only. We prac-

ticed strict phenotypic selection for low incidence of susceptible pustules (following seedling inoculation with mixed stem rust and leaf rust spores) combined with adult plant leaf; stem, and stripe rust field resistance, and witnessed a rapid change in the average levels of resistance in the base population.

The recurrent mass selection procedure can readily be executed on a large scale and with a modest budget. The program is surprisingly cost-effective, and in our opinion more so than conventional wheat breeding techniques such as pedigree, bulk, and doubled haploid. The labor required for producing 60,000–70,000 F_1 hybrid seeds is roughly equivalent to making about 150–200 planned crosses by hand in a medium-sized pedigree breeding program. The number of F_1 produced was always well beyond the numbers we could utilize in a season. In this program, there are no pedigrees to keep and inbred lines are simply numbered consecutively in the F_6. The strong emphasis that is placed on early generation selection means that lines with obvious defects are eliminated early on, making it possible to carry larger initial populations. Since only the male parents are field-tested, selection response for adaptation, quality, and yield will be halved; however, much of this effect will be offset by the inbreeding steps.

Application of the technique can be very varied—for example, it may be used as a breeding strategy in its own right, employing either a single base population or several different base populations. Such populations may be used to derive either inbred lines or land race cultivars. On the other hand, it may be used to supplement a conventional pedigree breeding program with the aim to optimally exploit and pyramid beneficial genes within a small group of well-adapted elite lines. Recurrent mass selection applications may also differ with respect to the composition of the base population. The base population may be genotypically highly variable and assembled from diverse and not necessarily adapted germplasm. This would typically be the case, where a new breeding program is being initiated and the breeder wishes to experiment with a wider range of variation. On the other hand, it may be possible to construct the base population in such a way that it will be uniform in some aspects, yet variable for other traits—for example, by combining near-isogenic lines carrying a range of diverse resistance genes in a genetic background that has good processing quality and agrotype. In such a population, screening for quality and agrotype is negated and the breeder can focus all his effort on the development of lines with complex resistance.

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Rotation Design: A Critical Factor for Sustainable Crop Production in a Semiarid Climate: A Review

Randy L. Anderson

Abstract The concept of "fallow" has been a prominent management tactic in semiarid regions of the world, enabling producers to compensate for low precipitation. However, fallow phases lead to soil degradation. For example, winter wheat (*Triticum aestivum* L.)-fallow with tillage has been used for decades in the semiarid steppe of the United States; organic matter levels in soils have declined almost 60%. Thus, producers in this region are concerned about the future sustainability of this rotation. No-till practices, however, improve water relations such that more crops can be added to the winter wheat-fallow rotation. This change in cropping patterns has led producers to seek cropping systems that are economically viable, restore soil health, improve resource-use-efficiency, and reduce the need for external inputs such as pesticides and fertilizers. Long-term rotation studies in the steppe show that continuous cropping with no-till can accrue these four goals. However, with water supply often being limiting, rotation design is critical for success with continuous cropping. Designing rotations in a cycle-of-four with a diversity of crops, increases net returns four-fold while reducing the cost of weed management 50% compared with conventional systems. Continuous cropping for 12 years increased soil organic carbon by 37% and nitrogen by 20% in the top 5 cm of soil, and also improved soil porosity and aggregate stability. Consequently, soil productivity has increased two-fold. Also, the cycle-of-four design provides a crop niche for legumes in this semiarid climate, which further enhances soil function. Some crops improve water-use-efficiency of the following crops by 20–35%, thus ameliorating the impact of low precipitation. Continuous cropping with no-till has initiated a spiral of soil regeneration.

Keywords Soil restoration · Crop diversity · No-till · Resource-use-efficiency

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1 Introduction

Winter wheat is the predominant crop grown in the central steppe of the United States an area in eastern Colorado and Wyoming and western Kansas, Nebraska, and South Dakota. It is grown in a winter wheat-fallow rotation to adjust for low precipitation, which ranges from 350 to 450 mm and occurs mainly from April through August. Neither crops nor weeds are allowed to grow during the fallow phase, therefore precipitation is stored in soil. Soil water gained during fallow periods reduces yield variability and crop loss due to drought stress.

But winter wheat-fallow has led to extensive soil degradation. Almost 60% of the original organic matter present in the soil has been lost (Bowman et al., 1990) and soil is especially prone to wind erosion during fallow periods (Peterson et al., 1993). A further aspect of winter wheat-fallow is its inefficiency in using precipitation for crop growth. Less than half the precipitation received during the two years is used by winter wheat; the rest is lost to evaporation, run-off, or leaching below the crop rooting zone (Farahani et al., 1998).

No-till practices preserve crop residue on the soil surface and improve water relations, allowing producers to add more crops to the winter wheat-fallow rotation (Peterson et al., 1996). Corn (*Zea mays* L.), proso millet (*Panicum miliaceum* L.), sunflower (*Helianthus annuus* L.), and dry pea (*Pisum sativum* L.) are now grown in sequence with winter wheat and fallow fields. This change in cropping practices has stimulated producers to examine their long-term goals with farming systems. Economics of more diverse rotations have been favorable (Dhuyvetter et al., 1996), but producers also want to repair the damage to soils caused by winter wheat-fallow.

In recent years, scientists and producers have contemplated sustainable cropping systems. Defining sustainability has been somewhat elusive, but producers in the steppe have four goals: economically viable rotations, that restore soil health,

improve resource-use-efficiency, and reduce need for external inputs. A goal of soil health is to increase the quantity of organic matter that subsequently improves nutrient cycling, soil aggregation, precipitation infiltration, water storage, and soil microbial functioning (Carter, 2002; Rasmussen and Collins, 1991). Soil productivity has been directly related to soil organic matter levels (Bauer and Black, 1994). Because water supply is limited in this semiarid region, producers would like to improve the water-use-efficiency of crops. They also would like cropping systems that are not so dependent on agrochemicals.

Several long-term rotation studies have been established in the steppe in the past 20 years, and trends with yield, economics, and soil changes across time have been quantified. These trends may provide insight for achieving these four goals. However, we are also intrigued by philosophical discussions related to sustainability. Hill and MacRae (1995), analyzing various approaches to sustainable systems, suggested redesigning cropping systems based on ecological principles rather than modifying existing systems in response to a specific issue. Brummer (1998) encouraged scientists to prioritize the design of sustainable systems, and then focus research on crop productivity within that design. This approach contrasts with the historical perspective of emphasizing the productivity of crops without regard to rotation design. Therefore, we also consider rotation design when evaluating trends with these rotation studies. Our assessment may provide insight for the development of sustainable systems not only in the U.S. steppe, but also in other semiarid regions of the world.

2 Biological Trends with No-till Cropping Systems

In the 1980s, long-term rotation studies were started in the central steppe of the United States at three sites in Colorado (Peterson et al. 1993; Anderson et al. 1999) and two sites in South Dakota (Beck, 2007; Stymiest et al., 2007). These studies compared various combinations of crops, ranging from winter wheat-fallow to continuous cropping; rotations with continuous cropping did not include a 12- to 14-month fallow period. Rotations included both cool- and warm-season crops. Cool-season crops were winter wheat, spring wheat, and dry pea, which are planted either in September or late March. Warm-season crops, planted in May or June, were corn, proso millet, sunflower, chickpea (*Cicer arietinum* L.), and soybean (*Glycine max* (L.) Merr.). The studies were established in Mollisol soils of the grass steppe. All phases of each rotation were included in each study. After several years of these studies, we examined yield, soil changes, and pest populations to identify production choices that favor sustainability with semiarid cropping systems.

2.1 Land Productivity and Economics

Adding summer crops, such as corn, to winter wheat-fallow increases land productivity. For example, annualized yield per land area can be almost doubled with some

rotations. Annualized yield is calculated by adding the yields of all crops in a rotation for a given year, then dividing by the number of years in the rotation. This value includes the investment of fallow periods in crop production. At a rotation study near Akron, Colorado, productivity of winter wheat-corn-fallow (W-C-F), winter wheat-corn-proso millet-fallow (W-C-M-F) or winter wheat-corn-proso millet (W-C-M), was two-fold greater than winter wheat-fallow (W-F). For example, the annualized yield of W-F was 970 kg/ha, contrasting with W-C-M-F producing 1,910 kg/ha—an increase of 97%. An intriguing trend was that continuous cropping, W-C-M, also yielded two-fold more than W-F. Similar results occurred at the other studies in the steppe; land productivity increased two-fold with rotations comprised of several crops (Peterson et al., 1993; Stymiest et al., 2007).

Rotations with more crops and less fallow time also improve economics. Net returns for diverse rotations were 25% higher compared with W-F throughout the central steppe (Dhuyvetter et al., 1996). Crop diversity in rotations also reduced financial risk.

2.2 Soil Restoration

After eight years with the Akron, Colorado study, soil organic carbon (SOC) increased 20% in the top 5 cm of soil with continuous cropping, compared with W-F (Fig. 1). However, SOC did not increase if a 12- to 14-month fallow period was included in a rotation, even when three crops were grown before a fallow period. In a second study in the region, 12 years of continuous cropping increased SOC by

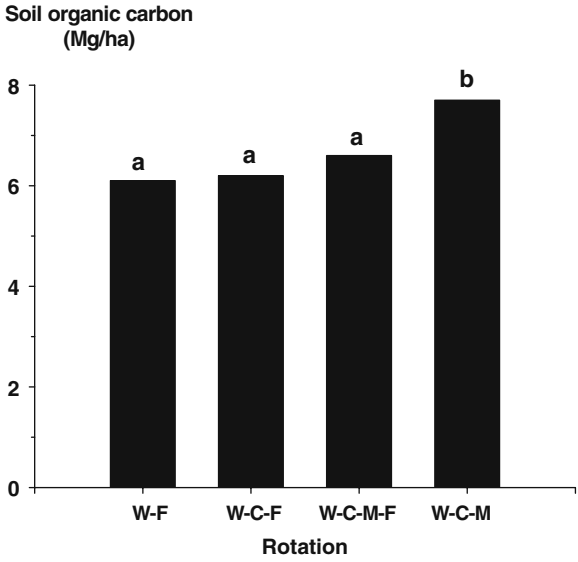

Fig. 1 Rotation design influences level of soil organic carbon in the 0–5 cm depth of soil. Abbreviations are: W = winter wheat; F = fallow; C = corn; M = proso millet. The conventional rotation in this region (W-F) included tillage, whereas W-C-F, W-C-M-F, and W-C-M were no-till. Data collected in the eighth year of the rotation study at Akron, Colorado (Adapted from Bowman et al., 1999)

37% as compared to W-F (Sherrold et al., 2003). At both studies, fallow fields in any rotation minimized the gain in SOC by continuous cropping.

Continuous cropping also increased aggregate stability compared with W-F in the Akron, CO study (Wright and Anderson, 2000). However, aggregate stability did not improve with any rotation that included fallow fields, even rotations such as W-C-M-F. Shaver et al. (2002) found a similar trend in another study; continuous cropping increased aggregate development and soil porosity, subsequently improving precipitation infiltration and water availability for crops. But improvement in soil structure was eliminated by fallow periods, even if fallow fields occurred only once in four years. In all studies, increasing crop residue production and preserving residues on the soil surface was essential for soil restoration.

Shaxson (2006) suggested that cropping systems designed to enhance the functioning of the microbial community will favor soil renewal. No-till cropping systems in the steppe are achieving this goal also; soil microbial biomass C is 70% higher in continuous cropping as compared with W-F (Sherrold et al., 2005).

2.3 Resource-Use-Efficiency

2.3.1 Water

No-till practices improve precipitation-storage efficiency (PSE) during fallow periods. With tilled systems, PSE is less than 30%, whereas no-till and crop residue preservation on the soil surface improves PSE to 40% (Peterson et al., 1996). Another trend with PSE in no-till fallow fields is that storage efficiency is highest over the winter, but lowest during the summer months (Tanaka and Anderson, 1997). Adding warm-season crops like corn improves PSE during the shorter fallow intervals to more than 50% (Farahani et al., 1998).

In addition to improving PSE during fallow periods, no-till and diverse crop rotations increase the amount of precipitation converted into crop yield. Winter wheat-fallow converts 40–45% of precipitation received during the two years of this rotation into grain (Farahani et al., 1998). In contrast, rotations such as W-C-M-F convert almost 60% of precipitation into grain, whereas conversion with continuous cropping is 75%. Continuous cropping improves the conversion rate by minimizing the inefficiency of fallow periods.

Crop diversity provides an additional benefit for water use; some crops improve water-use efficiency (WUE) of the crops that follow (Anderson, 2005b). Winter wheat produces 20–35% more grain with the same water use in W-C-F, compared to W-M-F or W-F. For example, winter wheat will yield 3,930 kg/ha in W-C-F with 300 mm of water use, whereas the yield will be 2,940 kg/ha in W-M-F (Fig. 2). A similar gain in WUE occurs when corn precedes proso millet; proso produces 20–25% more grain with the same water use in W-C-M as compared with W-M. A surprising trend, however, was that fallow periods eliminated this synergistic interaction between corn and proso millet. Proso millet WUE and yield were the same in W-C-M-F and W-M—approximately 20% less as compared to W-C-M. We are

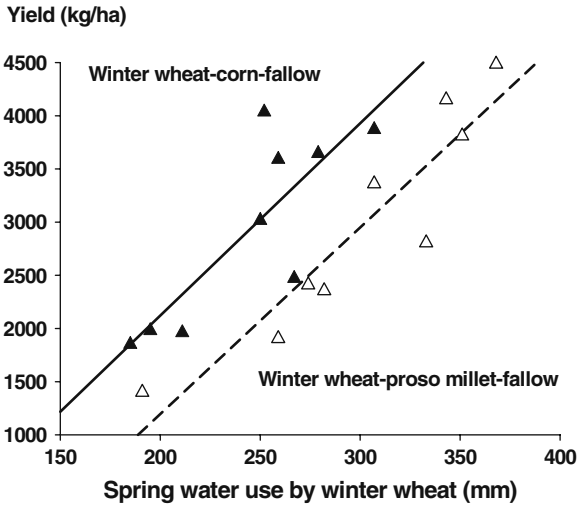

Fig. 2 Crop sequence can improve the grain yield-water use relationship for winter wheat. Two rotations are compared: winter wheat-corn-fallow (*black triangles* and *solid line*) and winter wheat-proso millet-fallow (*white triangles* and *dashed line*). Regression equation for winter wheat-corn-fallow was Y = 18.4X –1587, $r^2 = 0.67$; and for winter wheat-proso millet-fallow it was Y = 17.5X –2323, r^2 + 0.86. Data averaged across three years; study conducted at Akron, Colorado. (Adapted from Anderson, 2005b)

unable to explain why this synergistic trend occurs, but including corn in the rotation improves the WUE of other crops.

2.3.2 Nitrogen and Phosphorus

As found with SOC, continuous cropping increases soil organic N (SON) levels over time. Both Bowman et al. (1999) and Sherrold et al. (2003) reported that SON increased 15–20% with continuous cropping as compared with W-F. Also, both research teams found that a 12- to 14-month fallow period eliminated this gain in SON, even with rotations comprised of three crops and one fallow season.

Soils in the steppe with higher SON improve crop N-use efficiency. Maskina et al. (1993) found that corn yielded 10% more in high SON treatment as compared with a low SON treatment in a long-term crop residue study, even with adequate N fertilizer. The high SON treatment apparently increased the growth efficiency of corn, as the 10% yield difference remained regardless of the N fertilizer rates used.

A consequence of W-F has been the leaching of nitrates in the soil profile. Westfall et al. (1996) found that nitrate levels in the soil are lower in rotations with less fallow time; nitrate quantity in the upper 2 m of soil was 42% less in W-C-M-F as compared with W-F. In the Akron, Colorado study, nitrate quantity in the soil was further reduced by continuous cropping; in contrast, all rotations with fallow time favored nitrate accumulation and leaching in the soil profile (Anderson, 2005c). Zentner et al., (2002) reported similar results in the semiarid steppe of Canada; continuous cropping reduced nitrate leaching in soil as compared to rotations with fallow periods. They attributed less leaching in continuous cropping to greater synchrony between N release by mineralization and N uptake by the crop.

Rotation design also affects phosphorus-use efficiency. Bowman and Halvorson (1997) found that concentration of P in winter wheat was 13–30% greater in continuous cropping in comparison to rotations that included a 12- to 14-month fallow period. This trend was attributed to the recycling of P through plant residue; P is more available for plant uptake in the organic phase with plant biomass or organic matter. During fallow times, chemical reactions in soil convert P into inorganic forms that are less accessible for plants, whereas yearly contributions of plant biomass in continuous cropping favor the organic phase of P.

2.4 Pest Management

2.4.1 Root Diseases

Root diseases often reduce crop yield in the steppe (Cook, 1990). In the Akron, Colorado study, crop yield was related to how frequently the crop was grown in rotation (Anderson, 2005c). Grain yield of sunflower, corn, and winter wheat was 17–60% higher when grown once every four years, as compared with a cropping frequency of two years (Fig. 3). Diversity of crops in rotation reduces the severity of root diseases by disrupting the population dynamics of pathogens.

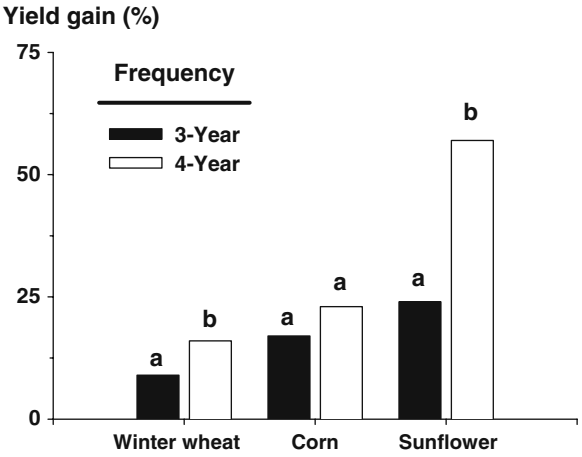

Fig. 3 Yield increases when a crop is grown less frequently than once every two years. Rotations compared for wheat (W-F, W-C-F, and W-C-M-F); for corn (M-C, W-C-F and W-C-M-F); and for sunflower (M-Sun, W-Sun-F, and W-C-Sun-F). Abbreviations are: W = winter wheat; F = fallow; C = corn; M = proso millet; and Sun = sunflower. Means reflect yield gain when compared to the crop in a two-year rotation. Data averaged across four years. Bars with an identical letter within a crop are not significantly different based on Fisher's Protected LSD (0.05). Means for all crops differed from a cropping frequency of two years. Study conducted at Akron, Colorado. (Adapted from Anderson, 2005c)

The drastic differences in yield with the frequency of sunflower is due to phoma (*Phoma macdonaldii* Boerma)—a fungi present in soil (Anderson et al., 1999). Phoma infects roots and the lower stem, reducing water and nutrient movement in the plant, and often causing plant lodging before harvesting. Bailey (1996) found a similar response of other oilseeds to the frequency of cropping in the semiarid steppe of Canada; she recommended growing oilseed crops once every four years.

A prevalent root disease of winter wheat is common root rot, caused by *Fusarium* and *Cochliobolus* fungi. For example, winter wheat yields are low following proso millet with W-M or W-C-M, yielding only 50% of winter wheat grown in W-C-M-F (Anderson et al., 1999). Wildermuth and McNamara (1991) found that proso millet is a host for the *Fusarium* and *Cochliobolus* species; common root rot severity in winter wheat following proso millet is similar to continuous winter wheat. Replacing proso millet with chickpea (a non-host legume) in the W-C-M rotation increased winter wheat yield by 28% across a seven-year interval (Stymiest et al. 2007).

2.4.2 Weed Management

Rotating cool- and warm-season crops helps weed management because different planting and harvest dates among these crops provide opportunities to prevent either plant establishment or seed production by weeds. The benefit of this strategy is related to weed seed survival in soil. With annual weeds, approximately 20% of the seeds are alive one year after seed shed, whereas less than 5% of their seeds are alive after two years. Rotating crops with different life cycles enables producers to favor the natural loss of weed seeds over time by preventing new seeds from being added to the soil.

However, rotation studies in the steppe show a surprising trend. Weed density declines over time when rotations are comprised of two cool-season crops followed by two warm-season crops (Anderson, 2008; Anderson and Beck, 2007). In contrast, weed density increases when rotations consist of one cool-season crop followed by one warm-season crop, such as W-M. Comparing trends across three rotation studies, weed density was six-fold greater in two-crop rotations as compared with rotations comprised of two cool-season crops followed by two warm-season crops (Fig. 4). Weed density in three-crop rotations was also higher than with four-crop rotations.

A second trend noted with these studies is that crops also need to differ within a seasonal interval of four-year rotations. For example, if two winter wheat crops were grown in succession for a cool-season interval, the density of winter annual grasses like downy brome (*Bromus tectorum* L.) escalated rapidly. In one study, downy brome density was forty times higher in four-year rotations with two years of winter wheat, as compared to rotations with a sequence of dry pea and winter wheat (Anderson et al., 2007). Dry pea is planted in late March, which provides an opportunity to control downy brome emerging over winter. A similar benefit is gained with crop diversity during the warm-season interval.

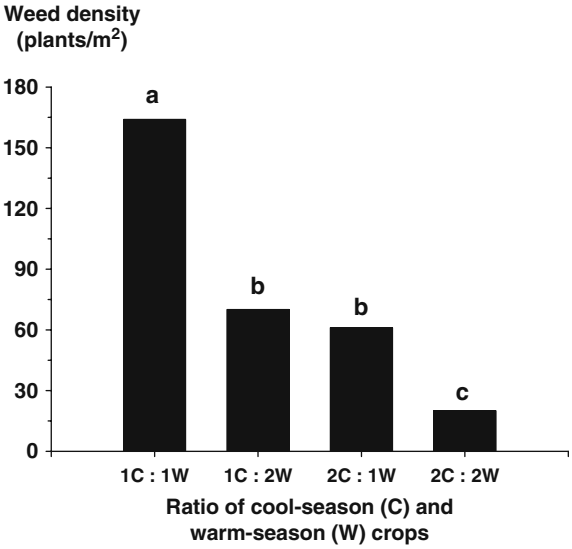

Fig. 4 Weed community density is affected by the ratio of cool-season to warm-season crops in rotations. Cool-season crops included winter wheat, spring wheat, and dry pea; warm-season crops are corn, proso millet, sunflower, chickpea, and soybean. Data averaged across three long-term studies in the U.S. steppe. Bars with an identical letter are not significantly different based on Fisher's Protected LSD (0.05). (Adapted from Anderson, 2008)

Weed management in these studies included conventionally-used herbicides, yet weed density was still affected by rotation design. Producers using rotations of two cool-season crops followed by two warm-season crops, such as Pea-W-C-M, are managing weeds with 50% less costs in comparison to rotations of fewer crops (Anderson, 2005a).

3 Rotation Design and Sustainability

One of our objectives with this assessment was to consider rotation design in relation to producers' goals for sustainability. Continuous cropping, such as W-M or W-C-M, is favorable for soil restoration and resource-use efficiency. However, these rotations have major limitations, especially with crop yield, residue production, and pest management. Winter wheat yield after proso millet is often less than 50% of yields after fallow periods. Root diseases are one cause of low yield, but a second reason is that it is difficult to convert more than 75% of precipitation into crop growth in this semiarid climate (Farahani et al., 1998). With W-M and W-C-M, average yields would require 85% of precipitation to be converted into crop growth (Anderson, 2005c).

Another limitation with W-C-M is that crop residue production by winter wheat is low, which reduces corn yield in the following year. Corn yields 15% less in W-C-M in comparison with W-C-M-F because of less favorable water relations with low residue quantities on the soil surface (Anderson et al. 1999). Furthermore, weed density escalates over time with W-M and W-C-M, and increases management costs (Fig. 4; Anderson, 2005a).

Designing rotations in a cycle-of-four, such as W-C-M-F, is favorable for land productivity and pest management. Grain yields of most crops are highest when grown once every four years (Fig. 3), whereas land productivity is two-fold greater than W-F. Weed density declines with the cycle-of-four design, enabling producers to reduce the cost of weed management (Anderson, 2005a). Based on these trends, we suggest that four-crop rotations may be the most favorable for achieving our four goals of sustainability, but only if crop sequencing can be developed for continuous cropping. The 12- to 14-month fallow period is detrimental for soil restoration, eliminating benefits gained by continuous cropping with SOC, SON, phosphorus uptake, aggregate stability, and soil porosity (Bowman et al., 1999; Wright and Anderson, 2000; Shaver et al., 2002; Sherrold et al., 2003). Also, fallow time leads to nitrate leaching in soil (Westfall et al., 1996; Anderson, 2005c). We question whether soil restoration and sustainability can be achieved in this region with any rotation that includes fallow periods.

3.1 Can We Replace Fallow Time with a Crop in this Semiarid Climate?

A concern with continuous cropping and eliminating the 12- to 14-month fallow period is water supply. For example, winter wheat can be planted two years in a row. However, grain yield and residue production of both winter wheat crops is 25–50% lower than wheat after fallow because of root diseases and inadequate water (Anderson, 2005c; Stymiest et al., 2007). A further consequence of this sequence is that weed density in winter wheat escalates rapidly (Anderson et al. 2007).

Legumes provide a more favorable option, especially for soil restoration. Legumes contribute N with its symbiotic production, which is especially valuable in the U.S. steppe, as most crops grown are cereals. Drinkwater and Snapp (2007) noted that in cereal-based rotations, legumes facilitate the accumulation of SOC and SON in soil; the higher level of SON improves the N uptake by following crops and reduces the need for fertilizers.

Furthermore, legumes provide flexibility for water management because they can be grown for forage or green fallow (terminated after six to eight weeks of growth) to adjust for water supply. A W-C-M-Pea (for forage) rotation uses approximately 75% of precipitation across four years for crop growth (Anderson, 2005c), near the conversion limit observed with continuous cropping in this region (Farahani et al., 1998). When dry pea is grown as green fallow in this rotation, precipitation use for crop growth is near 70%. Yet, even with short intervals of growth, dry pea or other legumes still increase SOC, SON, and soil microbial activity (Zentner et al., 2004; Biederbeck et al., 2005).

An additional benefit with legumes is higher yields of following crops. In one rotation study in the steppe, dry pea increased winter wheat yield by 5–15% compared with winter wheat after a fallow period (Beck, 2007). Yield increases because the WUE of winter wheat is 12% higher following dry pea as compared with fallow time (Fig. 5). In contrast, the WUE of winter wheat is 21% and 35% less following

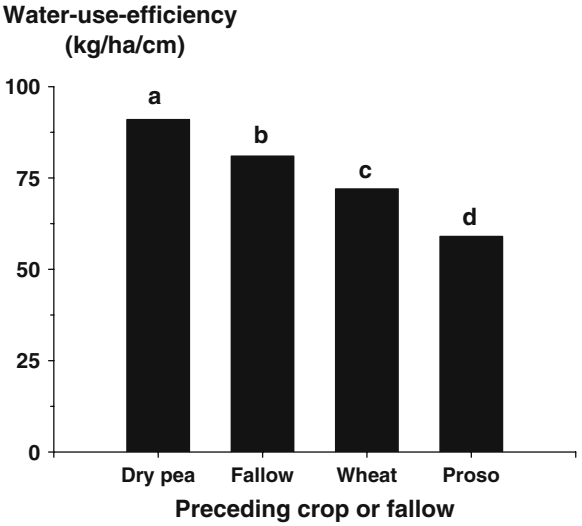

Fig. 5 Preceding crops affect the water-use-efficiency (WUE) of winter wheat. Data collected from rotational sequences of W-C-M-Pea, W-C-M-F, and W-C-M-W at Akron, Colorado. Abbreviations are: W = winter wheat; C = corn; M = proso millet; Pea = dry pea for forage; and F = fallow. WUE was defined as grain yield divided by total water use (growing precipitation plus soil water extraction). Data averaged across two years; bars with an identical letter are not significantly different based on Fisher's Protected LSD (0.05). (Adapted from Anderson, 2005c)

winter wheat and proso millet, respectively, as compared with dry pea as a preceding crop. Dry pea improves the WUE of winter wheat by suppressing root diseases (Cook, 1990) and favoring microbial interactions with winter wheat (Lupwayi and Kennedy, 2007). Winter wheat roots following dry pea are more readily colonized with mycorrhiza and contain more endophytic rhizobia; these microbial associations improve the plant's ability to withstand drought stress and to absorb nutrients. Also, Rice (1983) found that root exudates of dry pea improve the photosynthesis efficiency of cereal crops.

Dry pea as forage or green fallow also preserves the synergism with WUE between corn and proso millet, consequently increasing proso yields (Anderson, 2005c). Fallow eliminates this WUE synergism in the W-C-M-F rotation. The cycle-of-four design provides a niche for legumes in this semiarid region to improve crop yield and accelerate soil restoration.

3.2 Benefits of Rotations with the Cycle-of-Four Design

Planning rotations in a cycle-of-four allows producers to grow a diversity of crops with different water requirements and growth periods. Crop diversity is necessary for water management, especially to improve WUE and precipitation conversion to grain. Pest management and nutrient-use efficiency are also improved to

reduce production costs. Importantly, the cycle-of-four design with crop diversity can eliminate the need for a 12- to 14-month fallow period and enhance soil restoration with continuous cropping.

A striking change in yield occurs with soil restoration. When no-tillage was first used in the steppe, winter wheat yield increased by 20–30% as compared with tilled systems (Smika, 1990). Now, the yield potential of winter wheat is two-fold higher in no-tillage rotations (Anderson, 2005c). With W-F and tillage, winter wheat yield rarely exceeds 2,650 kg/ha. In contrast, wheat yields more than 5,400 kg/ha during favorable years with no-tillage and cycle-of-four rotations. Similarly, proso millet yield in some years exceeds 4,500 kg/ha in four-year rotations with no-tillage, whereas in W-M-F with tillage, proso rarely yields more than 2,000 kg/ha. Yields in no-tillage rotations greatly exceed the projected yields based on water supply and fertilizer inputs, demonstrating improved efficiency of the biological system.

Economic returns are also higher. In the early years of no-tillage, the net return was 25% higher in rotations with crop diversity as compared with W-F (Dhuyvetter et al., 1996). Now, net return is four-fold greater with no-tillage crop rotations (Anderson, 2007). Profit for W-F is $25/ha, whereas no-tillage rotations with crop diversity yield $100/ha. Improved economics reflect both higher land productivity and lower costs for weed management. Managing weeds costs $38/ha in no-tillage rotations arranged in the cycle-of-four, contrasting with $75/ha spent by producers to control weeds in W-F. Producers with cycle-of-four rotations are growing proso millet (Anderson, 2000) and winter wheat (Anderson, 2005a) without herbicides because weed density is so low.

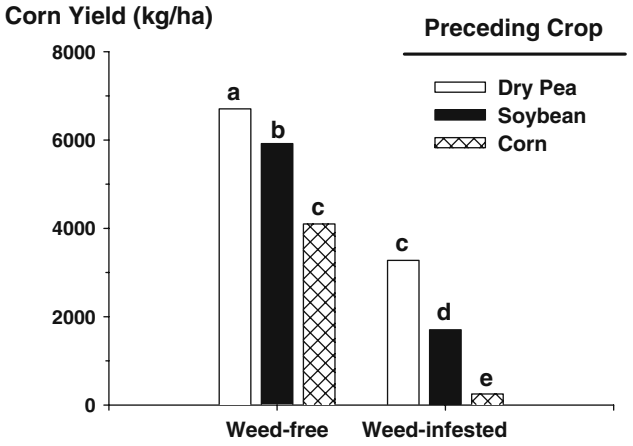

Fig. 6 Preceding crop influences yield of corn in both weed-free and weed-infested conditions. Study was established with no-till in the U.S. steppe. A uniform stand of foxtail millet represented the weed community in corn. Data averaged across two years; bars with an identical letter are not significantly different based on Fisher's Protected LSD (0.05). (Anderson R.L., research in progress)

Planning rotations with four crops is also effective in other climatic regions. For example, multifunctional rotations are used in the Netherlands to improve pest management, soil health, and nutrient cycling (Vereijken, 1992); maximum improvement occurs when rotations consist of at least four crops (Boller et al., 2004). This rotational design increases crop yield while reducing inputs with pesticides and fertilizers (Lewis et al., 1997).

Rotations with crop diversity may provide additional benefits. For example, we recently found that the preceding crop influences crop tolerance to weed interference. Corn tolerance to weeds in no-tillage is five-fold greater when following dry pea as compared to a corn monoculture (Fig. 6). Corn yield was also two-fold higher following dry pea as compared with soybean in weed-infested conditions. An intriguing trend was corn following dry pea, which yielded the same amount in weed-infested conditions as in continuous corn in weed-free conditions. Corn residues release toxins that damage corn seedling growth (Crookston, 1995), and reduce its competitiveness with weeds.

4 A Spiral of Soil Regeneration

No-tillage systems and residue management have transformed crop production in the semiarid steppe, doubling land productivity and reducing the need for fallow periods. Cropping patterns in the region also have changed; for example, dryland corn hectarage in Colorado increased from 4,000 ha in 1990 to more than 165,000 ha in 2000 (Anderson, 2005c).

Furthermore, soil health is being restored. Lal (2007) noted that removing crop residues from the farming system starts a spiral of soil degradation. No-tillage cropping systems in the U.S. steppe have reversed this spiral and are regenerating soil health (Anderson 2005c). Higher yields with four-crop rotations and no-tillage also increase crop residue production, which subsequently improves water relations to increase crop yields even more in following years. Thus, the system is self-perpetuating for soil restoration. As we gain more knowledge of beneficial interactions among crops, and improved soil functioning, we may be able to further accentuate this spiral of soil regeneration while alleviating the impact of low water supply. A key will be the design of rotations, especially if more crop sequences that are synergistic for resource-use efficiency can be identified.

Planning rotations in a cycle-of-four provide numerous benefits for producers in the semiarid steppe of the United States. We suggest that scientists and producers in other dry regions of the world may be able to gain a similar array of benefits with rotations comprised of several different crops and establish no-tillage practices.

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Parasitic Plants in Agriculture: Chemical Ecology of Germination and Host-Plant Location as Targets for Sustainable Control: A Review

Justin B. Runyon, John F. Tooker, Mark C. Mescher and Consuelo M. De Moraes

Abstract Parasitic plants are among the most problematic pests of agricultural crops worldwide. Effective means of control are generally lacking, in part because of the close physiological connection between the established parasite and host plant hindering efficient control using traditional methods. Seed germination and host location are critical early-growth stages that occur prior to host attachment, and provide promising targets for ecologically sound management of parasitic weeds. Knowledge of parasite-host interactions, particularly chemical cues that induce parasite seed germination and mediate host location, should facilitate the development of novel management approaches. In parasitic plants that attach to host roots—e.g., *Striga* and *Orobanche* spp.—seed germination is known to occur only in the presence of chemical stimulants released from plant roots. The recent finding that these same chemicals promote the colonization of beneficial fungi has potentially important implications for the control of parasitic plants. Far less is known about the early stages of parasitic plants that attach above-ground to host shoots—e.g., *Cuscuta* spp. Seeds of these parasites lack germination stimulants, and it was only recently shown that foraging *C. pentagona* seedlings use airborne cues to locate and select among hosts. We review research on seed germination and host location by the major parasitic weeds that attack agricultural crops, and discuss the implications of recent findings for the development of sustainable and effective management strategies.

Keywords *Striga* · *Orobanche* · *Cuscuta* · Strigolactones · Volatiles · Plant-plant communication

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1 Introduction

Approximately 4,500 species of flowering plants (more than 1% of all angiosperms) are parasitic, obtaining some or all of their water and nutrients from other plants (Kuijt, 1969; Nickrent, 2007). A small percentage of these parasitic species infest agricultural crops and cause serious problems for farmers in many parts of the world (Parker and Riches, 1993; Musselman et al., 2001). Few practical and economically sound methods are available for controlling parasitic plant species (Gressel et al., 2004; Rispail et al., 2007), in part because their physiological connection to host plants limits the usefulness of most herbicides. Parasitic weeds can also be difficult to eradicate because they often produce large numbers of long-lived seeds. For example, a single *Orobanche* sp. plant can produce over 200,000 dust-like seeds that remain viable for 8–10 years (Parker and Riches, 1993). In addition, parasitic plants that attack host roots can inflict serious damage to crop plants before the latter emerge from the soil, making it difficult to diagnose infestations before economic losses occur.

Breeding for host-plant resistance offers a potentially economical approach to controlling parasitic plants. However, with a few exceptions—e.g. resistance of cowpea to *Striga* (Lane et al., 1993)—breeding programs have not provided effective control and are challenging because plant resistance traits are often poorly characterized, genetically complex, and of low heritability (Rispail et al., 2007). Genetic engineering might help to overcome some of these difficulties (Bouwmeester et al., 2003), but societal concerns about genetically modified technology may prevent widespread adoption (Humphrey et al., 2006).

The search for improved or alternative approaches to controlling parasitic plants in agriculture will be facilitated by an increased understanding of the complex ecological and physiological interactions between parasitic plants and their hosts.

Host location is a critical part of the life cycle of the most damaging parasitic weeds, which are obligate parasites that depend on the limited reserves available in seeds to quickly locate suitable hosts. Host location thus seems a promising target for control strategies. In this paper, we review the most important plant parasites of agricultural crops, focusing on the chemical ecology of seed germination and host location, and discuss the potential for manipulating these mechanisms to control these important weeds.

2 The Major Parasitic Plants in Agriculture

Parasitism originated independently several times during angiosperm evolution, and the lifestyles of parasitic plants vary greatly across taxa (Kuijt, 1969; Nickrent et al., 1998). Some species are facultative parasites that are able to survive in the absence of hosts, while others are obligately parasitic and cannot develop independently. A distinction can be drawn between hemiparasitic plants that possess chlorophyll and are able to produce some of their required nutrients through photosynthesis and holoparasitic plants that lack chlorophyll and are completely dependent on host resources, but this distinction is not always clear-cut (Parker and Riches, 1993; Press and Graves, 1995). A more definitive division can be drawn between parasitic plants that make below-ground attachments to host-plant roots and those that attach above ground to host-plant shoots (Fig. 1). This review will focus on the most economically important groups of plant parasites: witchweeds, *Striga* spp. (Scrophulariaceae); and broomrapes, *Orobanche* spp. (Orobanchaceae), which attach to host roots; and dodders, *Cuscuta* spp. (Convolvulaceae), which make above-ground attachments to host shoots (Parker, 1991).

Striga spp. (Fig. 2) are obligate root hemiparasites and infest an estimated two-thirds of the cereals and legumes in sub-Saharan Africa, causing annual crop losses estimated at US$7 billion annually, and negatively influencing the lives of more than 300 million people (Berner et al., 1995; Musselman et al., 2001). Several species of *Striga* attack the major cereal crops in Africa (e.g., maize, sorghum, millet, and rice), but *S. hermonthica* and *S. asiatica* are the most widely distributed and destructive (Oswald, 2005). *Striga gesnerioides* parasitizes broadleaf plants and is a serious threat to cowpea production in many parts of Africa (Parker and Riches, 1993). In the 1950s, *S. asiatica* was discovered parasitizing maize in the southeastern United States, but its spread there has been halted by an intensive eradication program (Parker, 1991).

Orobanche spp. (Figs. 1 and 3) are obligate root holoparasites that constrain the production of many crops, primarily in the Mediterranean region, the Middle East, and northern Africa (Parker and Riches, 1993). Among the six *Orobanche* species considered serious pests, *O. ramosa* and *O. aegyptiaca* have the widest host ranges and heavily damage a variety of crops, including tomato, potato, eggplant, faba bean, lentil, peanut, chickpea, cucumber, cabbage, and sunflower (Parker and Riches, 1993). *Orobanche cumana* has a host range limited to Asteraceae, and it

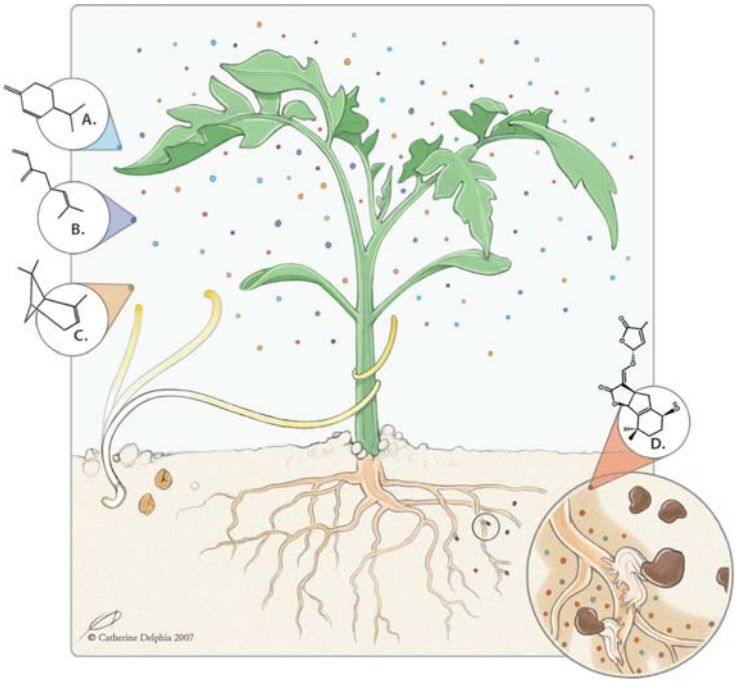

Fig. 1 Plant-derived chemical cues are used by both shoot and root parasitic weeds to locate hosts. Upon germination, the growth of *Cuscuta* seedlings is directed toward volatile compounds released from nearby tomato plants (*above-ground at left*). The entire blend of tomato volatiles (at least seven compounds) is most attractive, but three compounds from this blend individually elicit directed growth of *Cuscuta*: (**A**) β-phellandrene, (**B**) β-myrcene, and (**C**) α-pinene (Runyon et al., 2006). Seeds of the root parasites *Striga* and *Orobanche* will only germinate in response to specific chemicals released by plant roots (*below-ground at right*). These germination stimulants, called strigolactones, are active only within several millimeters of the host root. *Orobanche* seedlings are shown with haustoria attaching to the tomato (inset, lower right). Strigol (**D**) was the first germination stimulant identified. Strigol has not been isolated from tomato roots, but similar strigolactones are produced. The chemical ecology of host location by parasitic weeds provides early developmental points that could be exploited and manipulated for sustainable control

is an important pest of cultivated sunflowers (Parker and Riches, 1993; Press and Graves, 1995). Infestation by *Orobanche* spp. can result in total crop loss (Bernhard et al., 1998).

Cuscuta spp. have yellow-to-orange, rootless, leafless vines that attach to the shoots of host plants (Fig. 4). They are obligate holoparasites, typically exhibiting broad host ranges, and inflict serious damage to many crops, including forage legumes (alfalfa, clover, lespedeza), potato, carrot, sugar beets, chickpea, onion, cranberry, blueberry, and citrus (Dawson et al. 1994). Seeds of *Cuscuta* spp. have been transported worldwide in contaminated shipments of crop plant seeds. *Cuscuta pentagona* is a major weed of tomatoes in California, causing yield losses of

Fig. 2 A sorghum field infested with *Striga hermonthica* (*pink flowers*) in Ethiopia. *Striga* spp. attach to host-plant roots and produce green, flowering shoots that emerge 1–2 months later. Credit: Lytton J. Musselman

Fig. 3 *Orobanche ramosa* parasitizing cabbage in Sudan. *Orobanche* spp. attach to host-plant roots, and their flowering shoots, which lack chlorophyll, emerge several months later. Credit: Lytton J. Musselman

50–75% (Goldwasser et al., 2001). In China, several *Cuscuta* species inflict severe damage on soybeans (Dawson et al., 1994).

3 Parasitic Plants Use Chemical Cues to Locate Hosts

The seeds of most parasitic plants contain a few energy reserves that allow limited growth. Consequently, seedlings can survive only a few days after germination before attaching to a host. The imperative of finding hosts quickly presumably imposes strong evolutionary selection pressure favoring the development of efficient

Fig. 4 *Cuscuta pentagona* parasitizing tomato plants in California. *Cuscuta* spp. lack chlorophyll and attach above-ground to host-plant shoots. Credit: Jack Kelly Clark, courtesy UC Statewide IPM Program

host-location mechanisms. Both root and shoot parasitic plants utilize chemical cues released by host plants for this purpose (Fig. 1).

3.1 Root Parasitic Plants: Germination Stimulants

Seeds of *Striga* and *Orobanche* spp. germinate only in the presence of chemical compounds exuded from host roots (Fig. 1; Bouwmeester et al., 2007). Because these germination stimulants, collectively called strigolactones, are unstable and degrade rapidly in the soil, they occur at concentrations sufficient to induce germination only within a few millimeters of host roots (Fate et al., 1990). Concentration gradients of strigolactones may also facilitate directed growth of the parasite radicle toward the host root (Dubé and Olivier, 2001). The sensitivity of parasite seeds to these germination stimulants depends upon a conditioning period under warm and humid conditions and concomitant synthesis of gibberellins in seed tis-

sues (Matusova et al., 2004). To date, several germination stimulants have been isolated and identified from root exudates of both host and non-host plants. In work with *Striga lutea*, the first germination stimulant (strigol) was isolated from the root exudates of the non-host cotton (Cook et al., 1966). Strigol has since been found to be released by the roots of true hosts, including maize and millet (Siame et al., 1993). Additional strigolactone germination stimulants that have been identified include sorgolactone from sorghum, orobanchol and alectrol from red clover, and 5-deoxystrigol from *Lotus japonicus* (Hauck et al., 1992; Yokota et al., 1998; Akiyama et al., 2005). Recently, strigolactones have been shown to be apocarotenoids produced by plants via the carotenoid pathway, rather than sesquiterpenoids as had previously been assumed (Matusova et al., 2005). The details of germination induction by strigolactones are not understood (Bouwmeester et al., 2007), though possible mechanisms have been proposed (Mangnus and Zwanenburg, 1992). Application of ethylene can trigger seeds of *Striga* and *Orobanche* spp. to germinate, indicating that strigolactones may act by stimulating ethylene biosynthesis (Logan and Stewart, 1991). The recent discovery that strigolactones serve as important cues for plant-beneficial arbuscular mycorrhizal fungi (AMF; Akiyama et al., 2005; Besserer et al., 2006) suggests that parasitic plants may have co-opted these signals to recognize and locate host roots.

3.2 Shoot Parasitic Plants: Plant Volatiles

In contrast to root parasitic plants, germination of *Cuscuta* spp. seeds is not dependent on stimulants derived from a host plant (Dawson et al., 1994). Instead, seedlings must forage to locate potential hosts nearby. We recently reported that seedlings of *C. pentagona* use host-plant volatiles to guide host location and selection (Fig. 1; Runyon et al., 2006). It had previously been suggested that *Cuscuta* spp. seedlings forage randomly (Dawson et al., 1994), or orient their growth to various light cues associated with the presence of host plants (Benvenuti et al., 2005). While light cues may play a role in host location, we found that *C. pentagona* seedlings exhibited directed growth toward tomato volatiles experimentally released in the absence of any other plant-derived cues. Moreover, seedlings used volatile cues to "choose" tomatoes, a preferred host, over nonhost wheat. Several individual compounds from the tomato volatile blend were attractive to *C. pentagona* seedlings, including α-pinene, β-myrcene, and β-phellandrene, while one compound from the wheat blend, (*Z*)-3-hexenyl acetate, had a repellent effect. We subsequently confirmed that C. pentagona seedlings respond to volatiles from a range of host plants, including *Impatiens*, wheat (Runyon et al., 2006), and alfalfa (Mescher et al., 2006). These findings provide a plausible mechanism to explain previous reports of selective foraging by *Cuscuta* spp. (Kelly, 1990, 1992; Sanders et al., 1993; Koch et al., 2004). It is tempting to speculate that the remarkably similar but unrelated shoot-parasitic plants in the genus *Cassytha* (Lauraceae), and perhaps climbing

vines in general, might also use volatile cues to locate their hosts, but this possibility has yet to be examined empirically.

4 Control Strategies Targeting Germination/Host Location

Considerable research has examined the possibility of exploiting germination stimulants for control of *Striga* and *Orobanche*. Control strategies include: (1) inducing "suicidal germination," (2) inhibiting germination, and (3) reducing the production of germination stimulants by crop plants. In addition, the newly discovered role of strigolactones in the recruitment of symbiotic AMF (Akiyama et al., 2005) has opened new possibilities for modifying the production of germination stimulants by host plants. We are not aware of any studies exploring the possibility of disrupting host location by using *Cuscuta* spp., which have no germination stimulants. However, the recently documented role of volatiles in host location by *C. pentagona*, and the identification of several attractive and repellant compounds (Runyon et al., 2006), suggests that such strategies might be plausible.

4.1 Suicidal Germination

Inducing the germination of *Striga* and *Orobanche* spp. seeds in the absence of a suitable host plant results in "suicidal germination," and subsequent reduction in numbers of parasitic-plant seeds in soil. Both man-made and natural compounds have been investigated for their ability to induce germination. Analogs of strigol have been synthesized (e.g., GR 24 and Nijmegen 1) and are potent elicitors of germination in both *Striga* and *Orobanche* spp. (Wigchert et al., 1999); however, their instability in soil (Barbiker et al., 1987), and the high cost of producing large quantities of these compounds, have so far prohibited their use in agriculture (Humphrey et al., 2006). Ethylene has been a valuable component of the eradication program targeting *Striga asiatica* in the United States, where it induces about 90% germination when injected into the soil (Parker 1991). However, fumigating soil with ethylene is likely to negatively influence AMF and other nontarget soil microorganisms (Lendzemo et al., 2005). It has been proposed that ethylene-producing nonpathogenic bacteria could be used to induce suicidal germination of *Striga* (Berner et al., 1999), but a better understanding of bacteria/ethylene/crop interactions is needed before this method can be used in agriculture. Other natural compounds, including fungal toxins (Evidente et al., 2006) and methyl jasmonate (Yoneyama et al., 1998) have been shown to induce germination of *Striga* and *Orobanche* spp. seeds, but their potential uses in agriculture remain largely unexplored.

Planting nonhost trap crops that induce suicidal germination is perhaps the most effective strategy currently available for *Striga* control (Oswald, 2005). Recent studies in this area have focused on identifying and assessing the effectiveness of potential trap crops (Gbèhounou and Adango, 2003; Lins et al., 2006; Fenández-Aparicio et al., 2007; Khan et al., 2007) and the possibility of breeding for increased

production of germination stimulants (Botanga et al., 2003). Use of nitrogen-fixing legumes as trap crops has the added benefit of increasing soil fertility, which can further assist in *Striga* control because *Striga* thrive in poor soils (Parker and Riches, 1993). The efficacy of legume rotations could potentially even be improved by inoculating crops with supplemental nitrogen-fixing rhizobia, in combination with ethylene-producing bacteria, to simultaneously increase suicidal germination and soil fertility (Ahonsi et al., 2003; Babalola et al., 2007).

Legumes have also proven useful as part of a novel "push-pull" (stimulo-deterrent) pest management approach that illustrates the utility of increased plant diversity, simultaneously reducing *Striga* and lepidopteran stemborer infestations (Khan et al., 2000). Intercropping maize or sorghum with the leguminous trap crop *Desmodium* spp. decreases parasitism by *Striga* spp. and repels ovipositing stemborers, which subsequently move toward grasses bordering the field. *Desmodium* suppress *Striga*, not only by producing a germination stimulant, but also by producing chemicals that interfere with the development of haustoria (Khan et al., 2002).

4.2 Inhibiting Germination of Parasitic Plants

The sensitivity of *Orobanche* spp. seeds to germination stimulants is positively correlated with the production of gibberellin during seed conditioning; therefore, their germination can be inhibited by gibberellin biosynthesis inhibitors (Joel, 2000). Applying the gibberellin inhibitor uniconazole to soil near sunflowers significantly decreased broomrape parasitism and increased sunflower performance (Joel, 2000). Sunflower varieties that are resistant to *O. cernua* exude coumarins that inhibit germination and are toxic to newly germinated seedlings (Serghini et al., 2001). More recently, unidentified allelochemicals from oats appeared to inhibit seed germination of *O. crenata* and reduced parasitism when intercropped with legumes (Fenández-Aparicio et al., 2007). Seed germination can also be influenced by some amino acids, which have been shown recently to have profound effects on the development of *O. ramosa*. For instance, applying exogenous methionone almost completely inhibited seed germination and reduced the number of developing *Orobanche* spp. tubercles on tomato roots, possibly indicating that soil applications of amino acids or amino acid-producing microbes might be used to manage parasitic weeds (Vurro et al., 2006).

4.3 Reducing the Production of Germination Stimulants by Crop Plants

Decreased production of germination stimulants is the best characterized mechanism of crop resistance to parasitic plants (Rispail et al., 2007). This strategy has been exploited successfully in sorghum breeding to confer resistance of certain sorghum varieties to *Striga* (Haussmann et al., 2000). Resistance is apparently

absent in some crop plants, including cowpea and maize (Rubiales, 2003), although considerable variation has been reported among genotypes of tomato and *Arabidopsis* (Goldwasser and Yoder, 2001; El-Halmouch et al., 2006). Recent findings suggest that selecting for reduced production of germination stimulants might negatively influence crop interactions with beneficial AMF (Akiyama et al., 2005). Recognition that strigolactones that induce parasitic plant seeds to germinate also recruit nutrient-supplying AMF suggests that manipulating mycorrhizal colonization could be used to manage parasitic plants (Akiyama et al., 2005). Recent reports show that nutrient deficiency, which in some cases is mitigated by AMF, can increase strigolactone production by potential host plants (Yoneyama et al., 2007). Moreover, colonization of host plants by AMF can down-regulate the production of germination stimulants (Lendzemo et al., 2007; Bouwmeester et al., 2007), suggesting that enhancing AMF colonization of crop seedlings in fields could reduce strigolactone production, and possibly reduce the numbers of parasitic plant seeds that germinate.

4.4 Disruption of Volatile Host Location by Cuscuta *spp.*

The discovery that *Cuscuta* spp., like root-parasitic plants, use chemical cues to find hosts, may lead to control strategies aimed at disrupting host location analogous to those described for root-parasitic plants. Plant volatiles, even more so than strigolactones, are sensitive to environmental variables (De Moraes et al., 1998, 2001; Tooker and De Moraes, 2007; Tooker et al., 2008) and could potentially be manipulated (cf. Turlings and Ton, 2006) to reduce the attraction of *Cuscuta* spp. seedlings. In addition, the production of plant volatiles is a heritable trait (Degen et al., 2004) that could potentially be incorporated into a plant-breeding program for *Cuscuta* resistance. Moreover, because at least one repellent compound ([*Z*]-3-hexenyl acetate) has been identified, a "push-pull" approach for control of *Cuscuta* spp. can be envisioned similar to that used for African stemborers. However, little to no work to date has examined the feasibility of such approaches, and further work is needed to elucidate how *Cuscuta* spp. perceive and respond to plant volatiles.

5 Conclusion

In spite of intensive research, adequate strategies for controlling parasitic plants remain elusive, and these weeds continue to threaten agricultural crops worldwide. Chemically mediated interactions between early-stage parasitic plants and their hosts play a key role in infestation and may be exploited for control. Recent advances in this area suggest a number of potentially fruitful approaches, including the prospect of simultaneously managing beneficial symbionts and parasitic weeds. For example, implementing cultural practices that favor AMF, such as reducing tillage and fungicide application, could improve growth and increase drought tolerance in crops (Plenchette et al., 2005), and potentially reduce *Striga* infestations

(Lendzemo et al., 2007). Additional research is needed to understand the mechanisms underlying strigolactone perception and responses in both parasitic plants and AMF. Intercropping with nonhost plants that induce "suicidal germination" and/or are allelopathic to root parasites (e.g., Khan et al., 2002) is another promising approach that warrants continued efforts to identify potential trap crops and improve their efficacy. Recent work on the role of volatiles in host location by *C. pentagona* suggests that control strategies aimed at disrupting host location might be used against parasites that make above-ground attachments, but more work is needed in this area. It seems unlikely that any single method alone will provide long-term control of parasitic weeds. An integrative approach incorporating one or several methods targeting the chemistry used in host location by parasitic weeds is more likely to provide sustainable strategies that will minimize crop losses.

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Rice Seed Invigoration: A Review

M. Farooq, S.M.A. Basra, A. Wahid, A. Khaliq and N. Kobayashi

Abstract Rice (*Oryza sativa* L.) provides about 55–80% of the total calories for people in South Asia, Southeast Asia, and Latin America. Elsewhere, it represents a high-value commodity crop. Change in the method of crop establishment from traditional manual transplantation of seedlings to direct seeding has been adopted in many Asian countries in the last two decades, in view of rising production costs, especially for labor and water. Seed invigoration is ascribed to beneficial treatments, applied to the seeds after harvest but prior to sowing, that improve germination or seedling growth or facilitate the delivery of seeds and other materials required at the time of sowing. Many seed invigoration treatments are being employed in a number of field crops, including rice, to improve seedling establishment under normal and stressful conditions. The treatments used to invigorate rice seed include hydropriming, seed hardening, on-farm priming, osmopriming, osmohardening, humidification, matripriming, priming with plant growth regulators, polyamines, ascorbate, salicylicate, ethanol, osmolytes, coating technologies, and more recently presowing dry heat treatments. In the wake of the day-to-day increasing cost of labor and shortage of water, direct seeding approaches in rice cropping systems are the subject of intensive investigation throughout the world and offer an attractive alternative to traditional rice production systems. In this regard, seed invigoration techniques are pragmatic approaches to achieving proper stand establishment in the new rice culture. They help in breaking dormancy and improving seedling density per unit area under optimal and adverse soil conditions. Induction and *de novo* synthesis of hydrolases, such as amylases, lipases, proteases; and antioxidants such as catalases, superoxide dismutase and peroxidases are reported to be the basis of improved performance using these techniques. The rice seed priming can be performed by soaking simply in water, a solution of salts, hormones, osmoprotectants, matric strain-producing materials, and other nonconventional means. Despite certain limitations, such as water potential, oxygen and temperature, rice seed invigoration has

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been worthwhile in improving rice yield and quality. Nevertheless, in-depth studies are imperative for understanding the physiological and molecular basis of rice seed priming.

Keywords Direct seeding · Dormancy · Growth · Rice · Seed priming · Stand establishment · Stress tolerance · Vigor · Yield

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1 Introduction

Rice (*Oryza sativa* L.) is a staple food for more than half of the world population which provides about 55–80% of total calories for people in South Asia, Southeast Asia, and Latin America. In the rest of the world, it represents a high-value commodity crop. Global food security is confronted by escalating food demand and endangered by dwindling water availability. In this scenario, both farmers and researchers are devising strategies for water-wise crop production without compromising on yield (Gleick, 1993). Incessant and dedicated efforts of the researchers have lead to a new way of cultivating rice that requires lesser water than the conventional production system. In this newly devised method, rice is grown in aerobic soils using

supplementary irrigation like other cereals and aims for high yields (Huaqi et al., 2002).

Although aerobic rice is an attractive alternative to the traditional rice production system (Balasubramanian and Hill, 2002; Farooq et al., 2006d), poor stand establishment and high weed infestation are major constraints in its mass scale adoption (Farooq et al., 2006c, d, j). One major advantage of a traditional transplanting system is weed control, which would need special emphasis in aerobic rice culture (Farooq et al., 2006j). Many recent studies have dealt with improving germination and the subsequent growth in this crop. The age of nursery seedlings is one of the important determinants of seedling establishment in transplanted rice. Traditionally, seeds are used for growing nurseries in most parts of the world, which result in poor and erratic seedling growth. In a system of rice intensification, younger nursery seedlings are transplanted than the conventional transplanting system (Farooq et al., 2006i). The growth of rice nursery seedlings and, subsequently, their performance in transplanted culture can also be improved through seed priming (Farooq et al., 2006 h, 2007a, b). Seed priming is reported to increase the root proliferation that enhances nutrient and water uptake (Farooq et al. 2006 k). It improves the tolerance to low temperature (Naidu and Williams, 2004; Sasaki et al*.,* 2005), salinity (Ruan et al., 2003; Kim et al*.,* 2006) and drought (Harris and Jones, 1997; Du and Tuong, 2002) by enhancing the activities of antioxidants, including superoxide dismutase, catalase (Fashui, 2002; Deshpande et al., 2003), peroxidases, and glutathione reductase (Fashui, 2002). Moreover, priming reduced the levels of active oxygen species and plasma membrane permeability (Fashui, 2002). Although earlier reviews (Khan, 1992; Basu, 1994; Bray, 1995; Taylor et al., 1998; Welbaum et al., 1998; McDonald, 2000; Harris, 2006) dealt elegantly with seed invigoration in various crop species, no single review is available on rice. This review sums up the current work accomplished on rice seed invigoration.

2 Seed Invigoration Strategies

Seed invigoration techniques are value-added treatments applied on a given seed lot to improve its field performance. This term is often used interchangeably with seed priming. However, it is an umbrella term, which comprises many presowing techniques. Seed invigoration or seed enhancements are "post-harvest treatments to improve germination and seedling growth or to facilitate the delivery of seeds and other materials required at the time of sowing" (Taylor et al*.,* 1998). This definition includes four general methods (Fig. 1): presowing hydration treatments (Lee and Kim, 1999, 2000; Basra et al*.*, 2003, 2004, 2005a, 2006b; Farooq et al., 2004a; Farooq and Basra, 2005; Farooq et al., 2006b, e, 2007a, b, c), low molecular weight osmoprotectants seed treatments (Taylor et al*.*, 1998), coating technologies (Ross et al., 2000; Song et al., 2005) and, more recently, presowing dry heat treatment (Farooq et al*.,* 2004b, 2005b, d). These treatments focus on shortening the seedling emergence time and protecting the seeds from biotic and abiotic

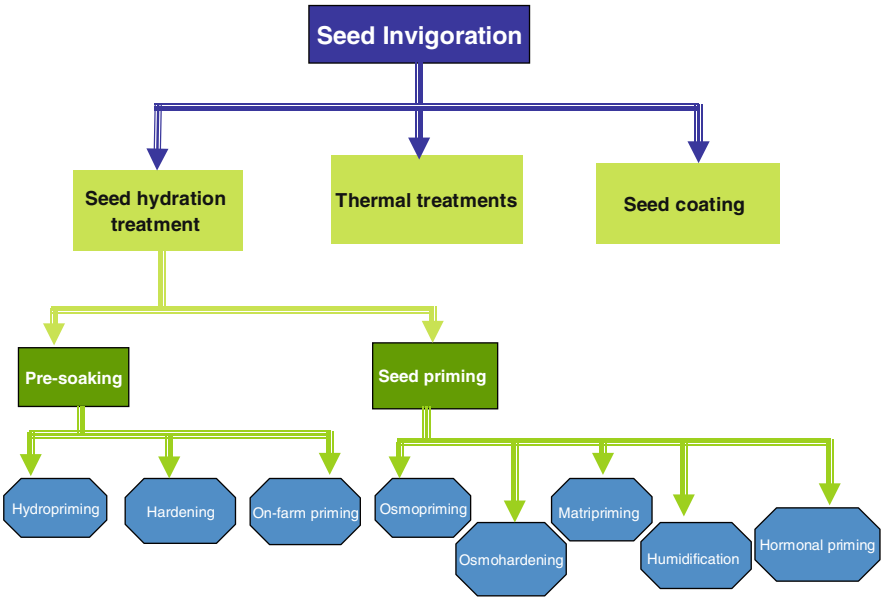

Fig. 1 Classification of seed invigoration techniques. Broadly, invigoration techniques can be divided into hydration, coating, and thermal treatments subdivided into Chilling treatment and Drought treatment. Seed hydration may be uncontrolled (presoaking) and controlled (seed priming). Depending on the nature of osmoticum used, seed priming may be osmopriming, osmohardening, humidification, matripriming, and hormonal priming

factors during critical phases of seedling establishment. Such treatments synchronize emergence (Tables 1, 2) and lead to uniform and vigorous stands and improved yield (Tables 3, 4).

2.1 Seed Hydration Treatments

Seed requires water, oxygen, and a suitable temperature for germination. Water uptake follows a triphasic pattern (Bewley, 1997). Phase I is imbibition, which commences with the physical uptake of water by the seeds, whether alive or dead. It is usually very rapid because the water potential difference between the dry seeds and water is usually great. In alive seeds, little metabolic activity occurs during this phase. In fact, dead seeds will imbibe water at the same rate as the viable ones. Phase II is the lag period. During this phase, there is little uptake of water, thus a little change in fresh weight, but considerable metabolic activity. The seed converts stored reserves (proteins, fats, and lipids) into compounds needed for germination. Phase III is radicle protrusion. This phase usually coincides with radicle emergence and is characterized by a period of rapid water uptake (a rapid increase in fresh weight). Seeds are desiccation-tolerant during Phases I and II, but frequently become intolerant during Phase III. Each phase of water uptake is controlled by water available to

Table 1 Efficacy of various seed priming treatments on the germination of rice

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Final germination percentage				
Hydropriming 48 h	Coarse	KS-282	36.67%	Farooq et al. (2006g)
Hydropriming 48 h	Fine	Super-Basmati	36.67%	Farooq et al. (2006g)
Hardening 24 h	Coarse	KS-282	16.70%	Basra et al. (2006b)
Hardening 24 h	Fine	Basmati-385	13.00%	Basra et al. (2005b)
Hardening 24 h	Fine	Super-Basmati	36.67%	Farooq et al. (2004a)
Hardening 24 h	Coarse	KS-282	13.67%	Farooq et al. (2004a)
Ascorbate priming 10 ppm	Fine	Super-Basmati	16.33%	Basra et al. (2006a)
Ascorbate priming 10 ppm	Coarse	KS-282	12.00%	Basra et al. (2006a)
Ascorbate priming 20 ppm	Fine	Super-Basmati	3.25%	Basra et al. (2006a)
Ascorbate priming 20 ppm	Coarse	KS-282	4.66%	Basra et al. (2006a)
Salicylicate priming 10 ppm	Fine	Super-Basmati	3.25%	Basra et al. (2006a)
Salicylicate priming 10 ppm	Coarse	KS-282	7.00%	Basra et al. (2006a)
Salicylicate priming 20 ppm	Fine	Super-Basmati	4.66%	Basra et al. (2006a)
Salicylicate priming 20 ppm	Coarse	KS-282	5.53%	Basra et al. (2006a)
Hardening 24 h	Fine	Super-Basmati	17.66%	Farooq et al. (2006a)
Osmohardening $CaCl_2$	Fine	Super-Basmati	11.00%	Farooq et al. (2006a)
Osmohardening NaCl	Fine	Super-Basmati	00.00%	Farooq et al. (2006a)
Osmohardening KNO_3	Fine	Super-Basmati	–11.34%	Farooq et al. (2006a)
Osmohardening KCl	Fine	Super-Basmati	11.00%	Farooq et al. (2006a)

Table 1 (continued)

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Mean germination time (MGT)				
Hydropriming 48 h	Coarse	KS-282	1.06 days	Farooq et al. (2006g)
Hydropriming 48 h	Fine	Super-Basmati	0.79 day	Farooq et al. (2006g)
Hardening 24 h	Fine	Basmati-385	0.47 day	Basra et al. (2005b)
Hardening 24 h	Fine	Super-Basmati	0.76 day	Farooq et al. (2004a)
Hardening 24 h	Coarse	KS-282	0.79 day	Farooq et al. (2004a)
Ascorbate priming 10 ppm	Fine	Super-Basmati	2.22 days	Basra et al. (2006a)
Ascorbate priming 10 ppm	Coarse	KS-282	2.25 days	Basra et al. (2006a)
Ascorbate priming 20 ppm	Fine	Super-Basmati	2.03 days	Basra et al. (2006a)
Ascorbate priming 20 ppm	Coarse	KS-282	1.88 days	Basra et al. (2006a)
Salicylicate priming 10 ppm	Fine	Super-Basmati	1.62 days	Basra et al. (2006a)
Salicylicate priming 10 ppm	Coarse	KS-282	0.85 day	Basra et al. (2006a)
Salicylicate priming 20 ppm	Fine	Super-Basmati	1.82 days	Basra et al. (2006a)
Salicylicate priming 20 ppm	Coarse	KS-282	0.88 day	Basra et al. (2006a)

Table 2 Efficacy of various seed priming treatments on the emergence of rice seedlings

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control (%/days/mg)	Reference
Final emergence percentage				
Hydropriming 48 h	Coarse	KS-282	44.18%	Farooq et al. (2006g)
Hydropriming 48 h	Fine	Super-Basmati	50.76%	Farooq et al. (2006g)
Hydropriming 48 h	Coarse	KS-282	23.34%	Farooq et al. (2006k)
Ascorbate priming	Coarse	KS-282	40.56%	Farooq et al. (2006k)
Osmohardening KCl	Coarse	KS-282	42.78%	Farooq et al. (2006k)
Osmohardening $CaCl_2$	Coarse	KS-282	33.15%	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	32.78%	Farooq et al. (2006k)
Hardening 24 h	Fine	Super-Basmati	46.78%	Farooq et al. (2004a)
Hardening 24 h	Coarse	KS-282	46.78%	Farooq et al. (2004a)
Ascorbate priming 10 ppm	Fine	Super-Basmati	28.77%	Basra et al. (2006a)
Ascorbate priming 10 ppm	Coarse	KS-282	21.99%	Basra et al. (2006a)
Ascorbate priming 20 ppm	Fine	Super-Basmati	18.58%	Basra et al. (2006a)
Ascorbate priming 20 ppm	Coarse	KS-282	21.99%	Basra et al. (2006a)
Salicylicate priming 10 ppm	Fine	Super-Basmati	31.83%	Basra et al. (2006a)
Salicylicate priming 10 ppm	Coarse	KS-282	30.23%	Basra et al. (2006a)
Salicylicate priming 20 ppm	Fine	Super-Basmati	29.83%	Basra et al. (2006a)
Salicylicate priming 20 ppm	Coarse	KS-282	19.87%	Basra et al. (2006a)
Hardening 24 h	Fine	Super-Basmati	38.82%	Basra et al. (2004)
Osmohardening $CaCl_2$	Fine	Super-Basmati	41.78%	Basra et al. (2004)
Osmohardening NaCl	Fine	Super-Basmati	35.56%	Basra et al. (2004)
Osmohardening KNO_3	Fine	Super-Basmati	25.34%	Basra et al. (2004)
Osmohardening KCl	Fine	Super-Basmati	30.78%	Basra et al. (2004)
Mean emergence time (MET)				
Hydropriming 48 h	Coarse	KS-282	2.87 days	Farooq et al. (2006g)
Hydropriming 48 h	Fine	Super-Basmati	2.97 days	Farooq et al. (2006g)

Table 2 (continued)

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control (%/days/mg)	Reference
Hydropriming 48 h	Coarse	KS-282	1.66 days	Farooq et al. (2006k)
Ascorbate priming	Coarse	KS-282	2.36 days	Farooq et al. (2006k)
Osmohardening KCl	Coarse	KS-282	2.85 days	Farooq et al. (2006k)
Osmohardening $CaCl_2$	Coarse	KS-282	2.34 days	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	2.30 days	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	1.2 days	Basra et al. (2006b)
Hardening 24 h	Fine	Basmati-385	0.5 day	Basra et al. (2005b)
Hardening 24 h	Fine	Super-Basmati	2.87 days	Farooq et al. (2004a)
Hardening 24 h	Coarse	KS-282	2.87 days	Farooq et al. (2004a)
Ascorbate priming 10 ppm	Fine	Super-Basmati	2.52 days	Basra et al. (2006a)
Ascorbate priming 10 ppm	Coarse	KS-282	2.28 days	Basra et al. (2006a)
Ascorbate priming 20 ppm	Fine	Super-Basmati	1.82 days	Basra et al. (2006a)
Ascorbate priming 20 ppm	Coarse	KS-282	2.28 days	Basra et al. (2006a)
Salicylicate priming 10 ppm	Fine	Super-Basmati	3.14 days	Basra et al. (2006a)
Salicylicate priming 10 ppm	Coarse	KS-282	0.08 day	Basra et al. (2006a)
Salicylicate priming 20 ppm	Fine	Super-Basmati	1.67 days	Basra et al. (2006a)
Salicylicate priming 20 ppm	Coarse	KS-282	0.51 day	Basra et al. (2006a)
Hydropriming 48 h	Coarse	KS-282	0.6 day	Farooq et al. (2006c)
Ascorbate priming	Coarse	KS-282	0.7 day	Farooq et al. (2006c)
Osmohardening KCl	Coarse	KS-282	1.9 days	Farooq et al. (2006c)

Table 2 (continued)

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control (%/days/mg)	Reference
Osmohardening $CaCl_2$	Coarse	KS-282	1.4 days	Farooq et al. (2006c)
Hardening 24 h	Coarse	KS-282	1.7 days	Farooq et al. (2006c)
Hydropriming 48 h	Fine	Super-Basmati	0.32 day	Farooq et al. (2006l)
Ascorbate priming	Fine	Super-Basmati	0.36 day	Farooq et al. (2006l)
Osmohardening KCl	Fine	Super-Basmati	0.89 day	Farooq et al. (2006l)
Osmohardening $CaCl_2$	Fine	Super-Basmati	1.75 days	Farooq et al. (2006l)
Hardening 24 h	Fine	Super-Basmati	1.55 days	Farooq et al. (2006l)
Hydropriming 48 h	Coarse	KS-282	0.78 day	Farooq et al. (2007b)
Ascorbate priming	Coarse	KS-282	2.05 days	Farooq et al. (2007b)
Osmohardening KCl	Coarse	KS-282	2.02 days	Farooq et al. (2007b)
Osmohardening $CaCl_2$	Coarse	KS-282	2.33 days	Farooq et al. (2007b)
Hardening 24 h	Coarse	KS-282	2.16 days	Farooq et al. (2007b)
Hydropriming 48 h	Fine	Super-Basmati	0.74 day	Farooq et al. (2007b)
Ascorbate priming	Fine	Super-Basmati	1.58 days	Farooq et al. (2007b)
Osmohardening KCl	Fine	Super-Basmati	1.58 days	Farooq et al. (2007b)
Osmohardening $CaCl_2$	Fine	Super-Basmati	1.98 days	Farooq et al. (2007b)
Hardening 24 h	Fine	Super-Basmati	1.89 days	Farooq et al. (2007b)

Table 3 Efficacy of various seed priming treatments on the seedling dry weight of rice

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Seedling dry weight				
Hydropriming 48 h	Coarse	KS-282	3.1 mg/seedling	Farooq et al. (2006k)
Ascorbate priming	Coarse	KS-282	2.77 mg/seedling	Farooq et al. (2006k)
Osmohardening KCl	Coarse	KS-282	4.5 mg/seedling	Farooq et al. (2006k)
Osmohardening $CaCl_2$	Coarse	KS-282	3.27 mg/seedling	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	3.1 mg/seedling	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	2.7 mg/seedling	Basra et al. (2006b)
Hardening 24 h	Fine	Super-Basmati	4.53 mg/seedling	Farooq et al. (2004a)
Hardening 24 h	Coarse	KS-282	4.35 mg/seedling	Farooq et al. (2004a)
Ascorbate priming 10 ppm	Fine	Super-Basmati	23.50 mg/seedling	Basra et al. (2006a)
Ascorbate priming 10 ppm	Coarse	KS-282	7.25 mg/seedling	Basra et al. (2006a)
Ascorbate priming 20 ppm	Fine	Super-Basmati	17.75 mg/seedling	Basra et al. (2006a)
Ascorbate priming 20 ppm	Coarse	KS-282	7.58 mg/seedling	Basra et al. (2006a)
Salicylicate priming 10 ppm	Fine	Super-Basmati	1.00 mg/seedling	Basra et al. (2006a)
Salicylicate priming 10 ppm	Coarse	KS-282	–6.00 mg/seedling	Basra et al. (2006a)
Salicylicate priming 20 ppm	Fine	Super-Basmati	3.75 mg/seedling	Basra et al. (2006a)
Salicylicate priming 20 ppm	Coarse	KS-282	–4.75 mg/seedling	Basra et al. (2006a)
Hydropriming 48 h	Coarse	KS-282	0.28 mg/seedling	Farooq et al. (2007b)
Ascorbate priming	Coarse	KS-282	0.91 mg/seedling	Farooq et al. (2007b)
Osmohardening KCl	Coarse	KS-282	0.97 mg/seedling	Farooq et al. (2007b)
Osmohardening $CaCl_2$	Coarse	KS-282	1.30 mg/seedling	Farooq et al. (2007b)
Hardening 24 h	Coarse	KS-282	1.28 mg/seedling	Farooq et al. (2007b)
Hydropriming 48 h	Fine	Super-Basmati	1.45 mg/seedling	Farooq et al. (2007b)
Ascorbate priming	Fine	Super-Basmati	1.12 mg/seedling	Farooq et al. (2007b)
Osmohardening KCl	Fine	Super-Basmati	2.03 mg/seedling	Farooq et al. (2007b)
Osmohardening $CaCl_2$	Fine	Super-Basmati	2.44 mg/seedling	Farooq et al. (2007b)
Hardening 24 h	Fine	Super-Basmati	2.26 mg/seedling	Farooq et al. (2007b)
Seed coating	Coarse	–	400–870%	Ross et al. (2000)

Table 4 Efficacy of various seed priming treatments on some agronomic traits and grain yield of rice

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Emergence to heading days				
Hydropriming 48 h	Coarse	KS-282	7.00 days	Farooq et al. (2006c)
Ascorbate priming	Coarse	KS-282	07.00 days	Farooq et al. (2006c)
Osmohardening KCl	Coarse	KS-282	13.00 days	Farooq et al. (2006c)
Osmohardening $CaCl_2$	Coarse	KS-282	11.00 days	Farooq et al. (2006c)
Hardening 24 h	Coarse	KS-282	11.00 days	Farooq et al. (2006c)
Hydropriming 48 h	Fine	Super-Basmati	7.67 days	Farooq et al. (2006l)
Ascorbate priming	Fine	Super-Basmati	10.76 days	Farooq et al. (2006l)
Osmohardening KCl	Fine	Super-Basmati	15.00 days	Farooq et al. (2006l)
Osmohardening $CaCl_2$	Fine	Super-Basmati	17.34 days	Farooq et al. (2006l)
Hardening 24 h	Fine	Super-Basmati	15.00 days	Farooq et al. (2006l)
Heading to maturity days				
Hydropriming 48 h	Coarse	KS-282	5.00 days	Farooq et al. (2006c)
Ascorbate priming	Coarse	KS-282	07.00 days	Farooq et al. (2006c)
Osmohardening KCl	Coarse	KS-282	10.00 days	Farooq et al. (2006c)
Osmohardening $CaCl_2$	Coarse	KS-282	08.00 days	Farooq et al. (2006c)
Hardening 24 h	Coarse	KS-282	09.00 days	Farooq et al. (2006c)
Hydropriming 48 h	Fine	Super-Basmati	4.00 days	Farooq et al. (2006l)
Ascorbate priming	Fine	Super-Basmati	4.33 days	Farooq et al. (2006l)
Osmohardening KCl	Fine	Super-Basmati	1.33 days	Farooq et al. (2006l)
Osmohardening $CaCl_2$	Fine	Super-Basmati	8.66 days	Farooq et al. (2006l)
Hardening 24 h	Fine	Super-Basmati	7.66 days	Farooq et al. (2006l)

Table 4 (continued)

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Grain yield				
Hydropriming 48 h	Coarse	KS-282	3.70%	Farooq et al. (2006c)
Ascorbate priming	Coarse	KS-282	11.11%	Farooq et al. (2006c)
Osmohardening KCl	Coarse	KS-282	18.51%	Farooq et al. (2006c)
Osmohardening $CaCl_2$	Coarse	KS-282	14.81%	Farooq et al. (2006c)
Hardening 24 h	Coarse	KS-282	11.11%	Farooq et al. (2006c)
Hydropriming 48 h	Fine	Super-Basmati	28.43%	Farooq et al. (2006l)
Ascorbate priming	Fine	Super-Basmati	24.64%	Farooq et al. (2006l)
Osmohardening KCl	Fine	Super-Basmati	30.80%	Farooq et al. (2006l)
Osmohardening $CaCl_2$	Fine	Super-Basmati	40.28%	Farooq et al. (2006l)
Hardening 24 h	Fine	Super-Basmati	30.33%	Farooq et al. (2006l)
Hydropriming 48 h	Coarse	KS-282	12.25%	Farooq et al. (2007b)
Ascorbate priming	Coarse	KS-282	14.52%	Farooq et al. (2007b)
Osmohardening KCl	Coarse	KS-282	21.93%	Farooq et al. (2007b)
Osmohardening $CaCl_2$	Coarse	KS-282	15.95%	Farooq et al. (2007b)
Hardening 24 h	Coarse	KS-282	14.24%	Farooq et al. (2007b)
Hydropriming 48 h	Fine	Super-Basmati	11.22%	Farooq et al. (2007b)
Ascorbate priming	Fine	Super-Basmati	19.64%	Farooq et al. (2007b)
Osmohardening KCl	Fine	Super-Basmati	25.26%	Farooq et al. (2007b)
Osmohardening $CaCl_2$	Fine	Super-Basmati	31.57%	Farooq et al. (2007b)
Hardening 24 h	Fine	Super-Basmati	25.61%	Farooq et al. (2007b)

the seeds (Taylor et al., 1998). Pre-sowing hydration techniques can be grouped into two categories depending on whether water uptake is uncontrolled or controlled.

2.1.1 Pre-Soaking

Presoaking includes the methods in which water is freely available to seeds, and its uptake is not restricted by the prevailing environment. The water uptake is governed by the affinity of the seed tissues to water. Common techniques include imbibing seeds on moistened blotters or soaking seeds in water. Important presoaking techniques that are employed to prime rice seed are detailed in the follow subsections.

a. Hydropriming

In hydropriming, seeds are soaked in water and dried before sowing (Soon et al., 2000). Soaking by submerging seeds in water can be performed with or without aeration (Thornton and Powell, 1992). Since an ample amount of water and oxygen and suitable temperatures are available, nondormant seeds would readily germinate. Because no chemicals are used during this process, it is an environmentally safe technique. A likely disadvantage of this technique is that the seed hydration is sometime uneven, which results in nonuniform germination (Pill and Necker, 2001).

Hydropriming duration is of vital importance for seed invigoration. To our knowledge, only one study was conducted to hydroprime rice for seed invigoration (Farooq et al., 2006 g). Coarse and fine rice seeds subjected to hydropriming for 12, 24, 36, 48, and 60 h in aerated tap water manifested improved vigor in both rice types except seeds hydroprimed for 60 h. Of these, maximum vigor improvement was noted in seeds hydroprimed for 48 h, which was followed by that of 36 h in both rice types. Improved germination and seedling establishment finally contributes towards the grain yield, thereby substantiating that hydropriming has the potential to improve germination and early seedling growth in coarse and fine rice (Farooq et al., 2006 g). In a field study, hydropriming for 48 h improved the emergence, seedling establishment, growth, and yield in direct-seeded coarse and fine rice cultivars (Farooq et al., 2006e, k). In another study, hydropriming for 48 h improved the growth of nursery seedlings and subsequently the growth, yield, and quality of both coarse and fine rice in transplanted cultures (Farooq et al., 2007a, b).

In nutshell, hydropriming can be employed to improve the performance of transplanted and direct-seeded rice. The best priming duration was 48 h for both rice types.

b. Hardening

Hardening, also called wetting and drying, or hydration-dehydration, refers to repeated soaking in water and drying (Pen Aloza and Eira, 1993). The hydration-dehydration cycle may be repeated twice, thrice, or for more times (Lee et al., 1998b; Lee and Kim, 2000). The beneficial effects of seed hardening are primarily related to pre-enlargement of the embryo (Austin et al., 1969), biochemical changes like enzyme activation (Lee et al., 1998a; Lee and Kim, 2000; Basra

et al., 2005a), and improvement of germination rate, particularly in old seeds (Lee et al., 1998a).

During seed hardening, the number of cycles and, above all, duration between the cycles are important for improving vigor. For rice seeds, two cycles of alternate wetting and drying are effective (Lee and Kim, 2000; Basra et al., 2003, 2004, 2005a). Hardening for 24 h proved best in vigor enhancement in both coarse (Basra et al., 2004) and fine rice (Farooq et al., 2004a). Seeds hardened for one and two cycles of 12 and 18 and 24 h enhanced vigor in both fine and coarse rice types, except the seeds hardened for 24 h (2 cycles) that behaved similarly to that of the control. Maximum vigor enhancement was noted in seeds hardened for 24 h (1 cycle), which was similar to that of seeds hardened for 12 h (Farooq et al., 2004a).

Seed hardening treatments have been found to be very effective in improving the germination and seedling stand establishment in rice (Lee et al., 1998a, Lee and Kim, 2000; Basra et al., 2003, 2004, 2005a; Farooq et al., 2004a; Farooq and Basra, 2005; Farooq et al., 2005a, 2006c, d, 2007a). Seed hardening was more effective for invigoration of normal and naturally aged rice seed than osmoconditioned ones (Lee and Kim, 2000; Basra et al., 2003). Mathew et al. (2004), after a series of laboratory and field experiments, established the superiority of the seed hardening strategy in improving the various attributes such as speed of germination, germination percentage, and seedling vigor that facilitated crop establishment in the field under subdued soil moisture. Seedling mortality was minimal and seedling density was higher in treatments involving hardening. Seed hardening for 24 h (1 cycle) also improved the growth, yield, and quality in direct-seeded coarse and fine rice types (Farooq et al., 2006l). In a separate study, seed hardening for 24 h not only improved the growth of nursery seedlings but also the subsequent growth, yield, and quality of both coarse and fine rice in transplanted culture (Farooq et al., 2007a, b). This suggests that seed hardening is an important approach in improving seed germination, stand establishment, and ultimately seed yield, when used up to 1 cycle of 24 h each (see Tables 1, 2, 3, 4).

c. On-farm seed priming

It is evident from the recent research that in a range of crop species, faster germination, early emergence, and vigorous seedling growth may result in high-yield crops by soaking in water for some time followed by surface drying before sowing referred to as "on-farm priming" (Harris et al., 1999, 2000; Musa et al., 1999). On-farm seed priming is a simple, low-cost, and low risk method for promoting seedling establishment, as well as vigorous and faster seedling growth. The duration of soaking is critical and should always be less than the safe limit (time to prime seed) for each crop cultivar. If the priming time exceeds that, it may lead to seed or seedling damage by premature germination (Harris et al., 1999). The concept of a "safe limit" differentiates on-farm seed priming from pregermination. Primed seeds will not germinate unless placed on a moist substrate, or unless moisture becomes subsequently available (e.g., rain). In contrast, seeds that have been soaked for longer than the safe limit will continue to

germinate even in the absence of an external moisture source. Use of pregerminated seed presents inherent risks, whereas primed seed behaves as dry seed if sowing is delayed or seedbed conditions are suboptimal.

Soaking overnight is also successful for rice (Harris et al., 2002). It is highly cost-effective for farmers, produces better stand; the crop matures earlier and gives higher yields for little cost. Primed rice seed germinates and seedlings emerge faster (1–3 d), more uniformly, and vigorously, leading to a wide range of phenological and yield-related benefits (Table 4). On-farm seed priming results in better emergence (91% vs 61%), earlier flowering (71 days vs 74.7 days), taller plants (108 cm vs 94 cm), longer panicles (22.4 cm vs 20.3 cm), and more numbers of panicles per plant (5.7 vs 4.9) in direct-seeded rice (Harris et al., 2002). For instance, in their experiment, Harris and Jones (1997) tested the germination response to the seed priming of 11 varieties of upland rice, including traditional and improved *O. sativa* and *O. glaberrima* varieties and new inter-specific hybrids. Seed priming with water for 24 h did not affect the final germination percentage, but it reduced time to 50% germination in all varieties from 46 h down to 32 h, which agrees well with the actual time saved by priming (7–20 h).

In summary, on-farm priming is a simple strategy for improving the phenology and yield of rice even under adverse soil conditions. Overnight soaking (before the actual radicle protrusion) is the best strategy in this regard.

2.1.2 Seed Priming (Controlled Hydration)

Seed priming is a technique by which seeds are partially hydrated to a point where germination-related metabolic processes begin, but radicle emergence does not occur (Heydecker and Coolbear, 1977; Bradford, 1986). During this process, seeds are placed in solutions with a high osmotic potential. This prevents the seeds from taking in enough water to enter Phase III of hydration. This actually results in an extension of Phase II, essentially restricting the seed within the lag phase (Taylor et al., 1998). During this period, the seeds are metabolically active and convert stored reserves for use during germination, wherein membrane and genetic repair is better than normal imbibition. The seeds are then removed from the priming solution, rinsed with water, and dried. Such seeds when planted show faster germination than the unprimed ones.

Primed seeds usually display increased germination rate, greater germination uniformity, and sometimes greater total germination percentage (Heydecker and Coolbear, 1977; Brocklehurst et al., 1984). These changes have been attributed to metabolic repair during imbibition (Burgass and Powell, 1984; Bray et al., 1989), a build up of germination-promoting metabolites (Farooq et al., 2006l), and osmotic adjustment (Bradford, 1986). However, for seeds that are not redried after treatment, a simple reduction in the lag time of imbibition takes place (Heydecker, 1977; Bewley and Black, 1982; Brocklehurst and Dearman, 1983; Bray, 1995; Taylor et al., 1998; Welbaum et al., 1998; McDonald, 2000). Seed priming can be accomplished by different means as detailed in the following subsections.

a. Osmopriming

The primary objective of employing seed osmopriming is to improve the germination and stand establishment. Osmoconditioning, osmopriming, or halopriming synonymous seed priming methods are used to describe the soaking of seeds in aerated low-water potential solutions to control water uptake and prevent radicle protrusion (Bray, 1995). Such treatments, followed by the dehydration of the seeds, have been demonstrated to improve the germination of numerous vegetable seeds, especially under suboptimal conditions (Heydecker et al., 1975; Brocklehurst and Dearman, 1983; Brocklehurst et al., 1984; Bradford, 1986; Bradford and Haigh, 1994; Karssen et al., 1989). In fact, osmoconditioned seeds are less sensitive to temperature and oxygen deprivation (Guedes and Cantliffe, 1980; Brocklehurst and Dearman, 1983; Corbineau et al., 1994). Of the different osmotica used as priming agents, polyethylene glycol, a variety of inorganic salts, proline, and mannitol are of special consideration.

Osmopriming with calcium chloride, potassium nitrate, sodium chloride, and polyethylene glycol-8000 improved the energy of germination and lowered mean germination time in rice (Ruan et al., 2002a). Priming with polyethylene glycol-8000 also accelerated the germination of coarse and fine rice (Basra et al., 2005a). In a greenhouse study, osmopriming with calcium chloride alone, and combined with sodium chloride, improved the seedling vigor index, and seedling and stand establishment of rice in flooded soil. The addition of gibberellic acid to a solution containing a mixture of calcium chloride and sodium chloride did not significantly promote either the speed of emergence or stand establishment as compared to the mixture of salts alone in solution (Ruan et al., 2002b). In a field experiment, rice seeds were primed with 4% potassium chloride before sowing, and 50 ppm paraquat sprayed at the tillering or booting stages. With seed priming, plant moisture content, leaf-area index, chlorophyll content, and nitrate reductase activity increased. Plant-moisture content and leaf-area index were the greatest when paraquat was applied at tillering, and chlorophyll content and nitrate reductase activity were greatest when paraquat was applied at booting (Sarma et al., 1993). Likewise, priming with lanthanum nitrate solutions can also accelerate the germination of the rice seeds, whilst significantly increasing seedling vigor in terms of root growth (Fashui et al., 2003; Zhang et al., 2005).

Researchers are trying to use different fertilizers for seed priming to improve their efficiency. In this respect, nutripriming with fertilizers to improve the performance of direct-seeded rice is another area of great interest (Du and Tuong, 2002). Kalita et al. (2002) found that nutripriming in 4% monoammonium phosphate resulted in the highest number of effective tillers and greater grain yield of direct-sown summer rice (Table 4). However, contrary to the above, micronutrient priming in a series of field experiments did not improve grain yield or the grain micronutrient content of rice (Johnson et al., 2005). In a laboratory study, fine and coarse rice seeds primed with urea, nitrophos, di-ammonium phosphate, and potassium sulphate resulted in a complete failure of germination and emergence. This was due to increased membrane damage (as seen from increased conductivity of seed leachates) with these fertilizers (Farooq et al., 2005c). This

suggests that a certain level of each fertilizer should be preoptimized before carrying out nutripriming.

Priming duration and salt concentration are of key importance and inversely proportional to each other. Priming seeds in higher concentrations of salts for longer durations may result in reduced and uneven germination and stand establishment. The performance of primed seeds is also dependent on the temperature during priming. For example, Lee et al. (1998c) concluded that priming of rice seed in distilled water (0 MPa) for 4 days at 15°C and 1 day at 25°C performed in a similar way, whereas four days was optimum time in polyethylene glycol solution (–0.6 MPa) regardless of the priming temperature. In another time-course study, osmopriming for 48 h was the most effective in both fine and coarse rice when seeds were soaked in aerated solutions of polyethylene glycol (ψ_s –1.25 MPa), while seeds osmopriming for 72 h behaved similar or inferior to untreated seeds, possibly due to priming for longer durations (Basra et al., 2005a).

Many researchers have reported improved germination and seedling stand establishment owing to a wide range of osmopriming protocols. Osmoconditioning (–1.1 MPa potassium nitrate solution) for 24 h improved germination and early seedling growth in fine (Basra et al. 2003, 2005b) and coarse rice (Basra et al., 2006a). Ruan et al. (2002a, b) reported a significantly enhanced energy of germination and declined mean germination time after osmopriming with calcium chloride singly and in combination with sodium chloride. Likewise, Lee et al. (1998c) found that osmopriming with –0.6 MPa polyethylene glycol solution at 25°C for four days took up to three days less time from planting to 50% germination, and improved the rate and final percentage of germination than those of untreated seeds. In addition to under optimal environmental conditions, the priming of rice seeds might be a useful way for better seedling establishment under adverse soil conditions (Lee et al., (1998a). Significantly greater and rapid germination of osmoprimed rice seeds under low temperature (5°C) and salt (0.58% sodium chloride) stresses were observed (He et al., 2002).

The ultimate advantage of osmopriming is yield enhancement (Table 4). In an adoption study in five states of Nigeria, 83 farmers out of the 300, who participated in the upland rice seed priming technology transfer between years 2000, and 2002 accrued 33–84% yield advantage by primed over nonprimed seeds. In view of this yield benefit, most of these farmers (94%) diffused the technology to their fellow farmers. This showed a wide acceptance of rice seed priming technology in the areas of coverage (Bakare et al., 2005).

In summary, a number of inorganic salts in appropriate concentrations can be used to improve germination in rice. Lowered osmotic potential of the solution appears to be instrumental in seed priming. The priming can be more beneficial if carried out up to 48 h; after that, it may lead to suboptimal germination and seedling stands.

b. Osmohardening

A new technique for rice seed invigoration has recently been introduced in which both seed hardening and osmoconditioning are successfully integrated—named

osmohardening (Farooq et al., 2006a). In this technique (like hardening), both the number and duration of cycles are important for improving the seed vigor. Because this is a relatively new technique, extensive work is imperative to find the most effective salts to be used as priming agents for rice seed invigoration. Available data show that a variety of salts were used to osmoharden coarse and fine rice. In a laboratory trial, both coarse and fine rice seeds were hardened (with water) and osmohardened (chlorides of calcium, potassium, sodium, and potassium nitrate solution) in such way that osmotic potential of all the solutions was –1.25 MPa. For both the rice types, osmohardening for 48 h with calcium chloride was better than other treatments followed by hardening and osmohardening with potassium chloride (Farooq et al., 2006a).

Osmohardening with calcium chloride was the most effective in improving the growth of rice nursery seedlings (Farooq et al., 2007b), and stand establishment in direct-seeded coarse and fine rice (Farooq et al., 2006a, c, d, k, 2007a, b, c). In fine rice, osmohardening with calcium chloride produced 2.96 t ha^{-1} (vs 2.11 t ha^{-1} from untreated control) kernel yield, 10.13 t ha^{-1} (vs 9.35 t ha^{-1} from untreated control) straw yield, and 22.61% (vs 18.91% from untreated control) harvest index (Farooq et al., 2006l). In coarse rice, on the other hand, osmohardening with potassium chloride produced greater kernel and straw yield, and harvest index, followed by that of calcium chloride hardening. Improved yield was attributed principally to the number of fertile tillers and 1,000 kernel weight (Farooq et al., 2006c). In another study, osmohardening with calcium chloride improved the initial seedling vigor and resulted in improved growth, yield, and quality of transplanted fine rice; the improvement in kernel yield was 3.75 t ha^{-1} (control: 2.87 t ha^{-1}), straw yield 11.40 t ha^{-1} (control: 10.03 t ha^{-1}), and harvest index 24.57% (control: 22.27%) (Table 4). The improved yield was attributed to the increase in the number of fertile tillers (Farooq et al., 2007b).

In essence, osmohardening is quite practical for enhancing the emergence, seedling stand establishment, growth, yield, and quality in both transplanted and direct-seeded rice. Osmohardening with calcium chloride was more effective for fine rice, and potassium chloride for coarse rice, in both culture methods. Further studies will provide the basis of enhancements in seedling density and economic yield in rice.

c. Matripriming

Matripriming involves controlled seed hydration similar to the natural moisture absorption of the plant media. Seeds are mixed into moist solid carriers such as granulated clay particles or vermiculite (Gray et al., 1990; Hardegree and Emmerich, 1992a, b). The surface of these compounds creates matrix forces that hold water to facilitate slow absorption by the seed (Taylor et al., 1998; Khan, 1992; Beckman et al., 1993). After treatment, the seed is separated from the solid carrier and allowed to dry.

Matripriming is a more effective vigor enhancement tool used in bold-seeded crops; the reason only very few studies have been conducted on small seeded crops such as rice. More recently, a matripriming method has been developed for rice using sand as a priming solid matrix (Hu et al., 2005). The seeds of four rice

varieties were mixed with sands that contained 3.8% (v/w) water and sealed in plastic boxes at 18°C for 72 h. This improved the emergence and seedling density of direct-sown rice in the laboratory. Moreover, seedling height, root length, number, and the dry weight of the root were significantly greater than the nonprimed controls. Field trials showed that the seed establishment and yield in matriprimed seeds were increased by 20–23% and by 10–31%, respectively (Table 4), as compared to soaked seeds without priming (Hu et al., 2005).

d. Priming with hormones and other organic sources

Improved seed performance has been achieved by incorporating plant growth regulators, polyamines, and certain other organic sources during priming and other presowing treatments in many vegetable and field crops, including rice (Kim et al., 1993; Jeong et al., 1994; Lee et al., 1999). Of the phytohormones, gibberellic acid is well-known to activate β-amylase for the breakdown of starch stored in seeds to be utilized by growing embryos during germination (Taiz and Zeiger, 2006). Both gibberellic acid and ethylene stimulate the elongation of mesocotyle, coleoptile, and internodes of rice seedlings after germination. Also, abscisic acid promotes elongation of the mesocotyle of rice seedlings (Kim et al., 1989; Lee et al., 1999). In rice, gibberellic acid treatment without seed priming enhances the time of seedling emergence by 1–2 days, depending upon gibberellic acid concentrations (Kim et al., 1993). In a study on kinetin and gibberellins applied to the dehusked seeds of indica and japonica rice under aerobic conditions, both these hormones stimulated the germination of rice. However, under anaerobic conditions, the effect of kinetin was negative while that of gibberellins was positive (Miyoshi and Sato, 1997a).

Studies of the individual or combined effects of gibberellic acid, urea, naphthaleneacetic acid, etc. on hybrid rice revealed that the application of 200 g of naphthaleneacetic acid ha^{-1} resulted in the improved percentage of emerged panicles while incurring the lowest cost. This treatment, along with 50 g gibberellic acid + 50 g naphthaleneacetic acid ha^{-1} and 100 g gibberellic acid ha^{-1}, recorded the maximum paddy yield. Based on the cost-effectiveness, naphthaleneacetic acid proved to be a viable alternative to gibberellic acid for hybrid rice seed production (Deshpande et al., 2003). Chen et al. (2005) soaked the seed of four rice cultivars in gibberellic acid and noted that seedling emergence and dry matter were increased significantly in some cultivars in response to seed gibberellic acid treatment.

Polyamines are known to have profound effect on plant growth and development (Watson and Malmberg, 1998). Polyamines, being cations, can associate with anionic components of the membrane, such as phospholipids, thereby stabilizing the bilayer surface and retarding membrane deterioration under stressful conditions (Basra et al., 1994). Conclusive evidence is available about the involvement of polyamines accumulation in the protection of plants against various environmental stresses (Bouchereau et al., 1999). Fine rice seeds soaked in lower (10 and 20 ppm) concentrations of polyamines (spermidine, putrescine, and spermine) displayed earlier, synchronized, and enhanced germination. Improvement in shoot and root length, seedling fresh and dry weight, and root and leaf

score was also observed. Seed treatment with 10 ppm putrescine solution was highly effective for most of the studied attributes (Farooq et al., 2008).

Salicylicate is an endogenous growth regulator of phenolic nature, which participates in the regulation of physiological processes in plants (Raskin, 1992). These include effects on ion uptake, membrane permeability, etc. (Barkosky and Einhelling, 1993). In addition, salicylicate interacts with other signaling pathways including those regulated by jasmonic acid and ethylene (Szalai et al., 2000, Ding and Wang, 2003). It also induces an increase in the resistance of seedlings to osmotic stress (Borsani et al., 2001), low or high temperature by activation of glutathione reductase and guaiacol peroxidase (Kang and Saltveit, 2002). Studies on the coarse rice seed priming with salicylicate resulted in greater vigor enhancement as compared with the control group. However, a prompt and most uniform germination and emergence was observed in seeds primed with 10 ppm ascorbate solution (Basra et al., 2006a). In a study, presowing seed treatments with 10, 20, and 30 ppm salicylicate resulted in earlier, synchronized, and enhanced germination. Improvement in root length, leaf score, and seedling fresh and dry weight was also recorded with these treatments, although 30 ppm concentration was the most effective (Farooq et al., 2007c).

Ascorbate is one of the most important antioxidants. Quite a few reports highlight the role of ascorbate in improving germination of cereals, including wheat, barley, and rice at lower concentrations (Naredo et al., 1998). In a laboratory study, it was revealed that priming with ascorbate at various (10–50 ppm) concentrations improved the germination and early seedling growth in both coarse and fine rice types, although priming with 10 ppm was the most effective (Basra et al., 2006a). Ascorbate priming also improved the growth, yield, and quality in direct-seeded coarse and fine (Farooq et al., 2006l) rice. In another study on transplanted rice, ascorbate priming not only improved the growth of nursery seedlings, but also the yield and quality of both coarse and fine rice types (Farooq et al., 2007a, b).

Among other organic sources, butenolides are a class of lactones with a four-carbon heterocyclic ring structure (Joule and Mills, 2000). The most common and important example of a butenolide is ascorbate. Butenolide derivatives are produced by some plants upon exposure to high temperatures, and these compounds can trigger seed germination in plants whose reproduction is fire-dependent (Flematti et al., 2004). In a recent study, low concentrations of butenolide greatly promoted seedling root and shoot length, and the number of lateral roots. The vigor index of smoke-water (1:500) and butenolide-treated rice seeds were significantly greater than that of untreated seeds (Kulkarni et al., 2006).

Effectiveness of imidacloprid (an insecticide) as a priming agent to improve yield has also been explored (Mathew et al., 2004). In a study, priming with imidacloprid, sodium chloride, potassium chloride, and *Azospirillum*, the imidacloprid greatly improved the seedling density and yield performance of rice seeds over the rest of the treatments (Mohanasarida and Mathew, 2005).

Ethanol has been reported to have stimulatory effects on the germination of seeds in many plant species (Taylorson and Hendricks, 1979; Bewley and Black, 1982). Farooq et al. (2006f) soaked fine rice seeds in 1, 5, 10 and 15% (v/v)

aerated solutions of ethanol for 48 h. None of the seeds could germinate at 10 and 15% (v/v) ethanol concentration, while 1 and 5% concentrations prompted a more uniform seedling emergence followed by more number of leaves per plant at 1% concentration. In another study, the inhibition of germination caused by de-husking japonica rice was overcome by 0.5–5% ethanol (Miyoshi and Sato, 1997b).

In short, priming with plant growth regulators and various other organic sources in relatively lower concentration has the potential to further enhance the uniformity in germination, stand establishment, growth, and harvestable yield.

e. Priming with low molecular weight osmolytes

Osmolytes help to maintain cyptoplasmic turgor pressure during water stress, stabilize the structure and functions of certain macromolecules, and ultimately promote the growth of plants under stressful conditions (Mickelbart et al., 2003). It is well-established that seed treatment and foliar application of these solutes might have some advantages, as they improve the tolerance ability of plants (Agboma et al., 1997).

In several temperate rice-growing countries of the world, the prevalence of low temperature at sowing results in poor rice seed germination, seedling establishment and vigor. Seeds of four rice cultivars (Sasanishiki H433, HSC-55, and Doongara) were soaked in various combinations of glycinebetaine in Petri dishes placed in a low-temperature glasshouse (18/13°C; day/night) for two days. After this soaking period, seedling emergence was faster in cold tolerant cultivar HSC-55 as noted from mean emergence time, while the other three cultivars (noncold-tolerant) displayed reduced seedling emergence, implying that glycinebetaine was ineffective on the latter cultivars. This indicated significant differences in the responses of genotypes to seedling emergence and vigor towards applied gibberellic acid and glycinebetaine under low temperature (Chen et al., 2005).

f. Humidification

Humidification is a presowing, controlled hydration treatment in which seeds are equilibrated under conditions of high humidity (Perl and Feder, 1981; Finnerty et al., 1992). In this technique, seeds are in direct contact with water vapor (Khan, 1992). To our knowledge, only one study was conducted to investigate the possibility of rice seed invigoration by this means. Humidification of normally germinating rice seeds did not increase germination under favorable conditions, but was accelerated in unfavorable soil and suboptimal temperatures (Lee et al., 1998a). Aged seeds humidified at 60% relative humidity showed no effect on germination rate or time to 50% germination. However, 80% relative humidity reduced the germination percentage and enhanced the time to 50% germination (Lee et al., 1998a).

2.2 Other Seed Invigoration Tools

Certain nonconventional means have been successfully used to invigorate rice seed to accomplish optimal seedling density and ultimate yield per unit area.

2.2.1 Thermal Treatments

The dry-heat treatment of seeds is used for two purposes one is to control the external and internal seed-borne pathogens, including fungi, bacteria, viruses, and nematodes (Nakagawa and Yamaguchi, 1989; Fourest et al., 1990); and the other is to break the dormancy of seeds (Zhang, 1990; Dadlani and Seshu, 1990). In general, high temperature in dry heat treatment reduces seed viability and seedling vigor, but optimum temperature for breaking dormancy promotes rice seed germination and seedling emergence (Lee et al., 2002). In a study on coarse and fine rice seeds, dry-heat treatment at 40°C for 72 h shortened the time to 50% germination and improved germination index, radicle and plumule length, root length, root/shoot ratio, root fresh and dry weight, radicle and plumule growth rate, and shoot fresh weight in fine rice. In coarse rice, none of these treatments improved germination and seedling vigor (Farooq et al., 2004b). In a laboratory study, coarse and fine rice seeds were exposed to thermal hardening (heating followed by chilling followed by heating and vice versa; and heating followed by chilling and vice versa). In fine rice, the heating–chilling–heating cycle was the best, while in coarse rice, the chilling–heating–chilling cycle performed better than all other treatments (Farooq et al., 2005b).

2.2.2 Seed Coating

Seeds vary greatly in their size, shape, and color. In many cases, seed size is small, making singularization and precision placement difficult. In addition, seeds should be protected from a range of pests that attack germinating seeds or seedlings. Seed-coating treatments can be employed in both situations; they can facilitate mechanical sowing to achieve uniformity of plant spacing, and can be applied in target zones with minimal disruption to the soil ecology and environment. Ross et al. (2000) found that rice seedling emergence was depressed by 40–60% when seeds were coated with a single super phosphate, mono ammonium phosphate, or potassium phosphate. By contrast, seed coating with rock phosphate did not affect final emergence, although it delayed seedling emergence by 2–3 days. Twenty days after sowing, coatings increased shoot dry weight, but decreased root dry weight of seedlings. The effect of coating treatments persisted up to 40 days after sowing, and at this stage, plant growth in terms of root length and dry weight and shoot dry weight increased by 400–870% (Table 3). Coating rice seeds with rock phosphate may be more promising in stimulating early rice growth on low P soils (Ross et al. 2000). Song et al. (2005) reported that film coating of rice seeds may improve the performance of direct-sown rice.

In Japan, coating rice with a source of oxygen (as CaO) has been practiced for decades to promote seedling emergence of direct-seeded rice in flooded soil (Ota and Nakayama, 1970). If seeds are broadcast in standing water, they remain floating due to lower specific gravity and tend to be poorly anchored, leading to floating or lodged seedlings. Yamauchi (2002) reported that the specific gravity of rice seeds can be increased by iron coating, which increases seed germination and thus stand establishment.

3 Factors Affecting Seed Priming

Many environmental variables in the priming protocols have different effects on the physiology of seed performance (McDonald, 2000). However, Corbineau and Côme (2006) opined that among the factors affecting seed priming, oxygen, temperature, and water potential of priming medium are the most important ones.

3.1 Oxygen

Oxygen has been identified as one of the most important variables modulating the effectiveness of seed priming. To the best of our knowledge, little information is available on the effects of aeration on rice seed priming and its subsequent performance. In a study, osmopriming in an aerated solution of polyethylene glycol solution with osmotic potential of –1.25 MPa improved germination and early seedling growth (Basra et al., 2005a). Osmohardening in aerated solutions of calcium chloride and potassium chloride, each with osmotic potential of –1.25 MPa, improved the germination, stand establishment, growth, and yield in transplanted (Farooq et al., 2007a, b) and direct-sown rice (Farooq et al., 2006c, k).

3.2 Temperature

Low temperatures during priming can change the seed performance (Lee et al., 1998c). This may delay the physiological processes of germination, even though the seed absorbs water in optimal amounts. Lower temperatures also reduce the possibility of microbial contamination during priming. Lee et al. (1998c) primed rice seeds at 15°C and 25°C, and the seeds were germinated at 17°C, 20°C, or 25°C. Considering germination rate, the optimum priming duration in water was four days at 15°C and one day at 25°C. However, priming in –0.6 MPa polyethylene glycol solution at low temperature did not affect the effectiveness of seed priming. Further studies are necessary to establish whether low, high, or optimum temperatures have any specific roles for enhanced germination in response to seed priming.

3.3 Water Potential

Seeds germinate when water potential reaches a critical level in the seed. This varies within and between plant species, but generally occurs when the seed environment is between 0 and –2 MPa (McDonald, 2000; Corbineau and Côme, 2006). Exceptions occur when seeds have impenetrable seed coats, or contain dormancy-causing chemicals that must be removed before germination occurs. Seeds having permeable seed coats usually go through three distinctive phases of germination: (1) imbibition; ψ_w of the seed environment is higher than that in the seed, causing water molecules to flow through the seed epidermis into the embryo, leading to (2) the activation phase; in which stored seed hormones and enzymes stimulate physiological

development leading to (3) growth of the radical; ending the germination phase (Taylor et al., 1998). Dormant (dry) seeds are usually at very low water potential, in the range of –350 to –50 MPa. Some metabolism occurs even at these low water potentials. Water movement into dry seed during the imbibition phase is rapid at first, but slows as the water potential of the seeds approaches that of the environment. If imbibition is too rapid (from an environment where water potential is very high), damage to hydrating cells often occurs (Soon et al., 2000; Pill and Necker, 2001).

Many researchers have reported improved germination and seedling stand establishment due to wide-range water potentials. Osmoconditioning with KNO_3 and water potential at –1.1 MPa improved germination and early seedling growth in coarse (Basra et al., 2006a) and fine rice (Basra et al., 2003, 2005b). Likewise, Lee et al. (1998c) found that osmopriming with –0.6 MPa polyethylene glycol improved the rate and final percentage of germination, and that rice seeds primed in this solution at 25°C for four days took lesser time from planting to 50% germination than that of untreated seeds (Lee et al., 1998c).

4 Mechanism of Rice Seed Priming

4.1 Physiological and Biochemical Basis

Seed vigor enhancement initiates the very early stages of germination, but not those associated with radicle growth. Still, fundamental understanding of the physiological and biochemical mechanisms of priming as to how they affect seed germination is elusive. For priming to be generally successful, seed moisture content should be maintained at 40–45% on a fresh weight basis, or about 90–95% of the seed moisture content necessary for germination (Gray et al., 1990). Priming of seeds triggers changes in the activities of enzymes, leading to changes in the levels of germination substrates (Fig. 2). These are discussed for rice in the following subsections.

4.1.1 Enzymes

Seeds represent a well-defined system as a sink, where resources are utilized for the production of seedlings. Rice seeds store starch, storage proteins, and a small amount of oils in the endosperm. Hydrolytic enzymes are mainly responsible for the hydrolysis of these reserves into the useable and readily available source of energy for embryo growth. Seed priming is reported to modulate enzymes of carbohydrate metabolism (Kaur et al., 2000, 2002), thereby increasing the available food for the growing embryo.

Studies on rice showed that priming increases the activity of hydrolytic enzymes (Table 5) and counteracts the effects of lipid peroxidation. During priming, *de novo* synthesis of α-amylase has been documented (Lee and Kim 2000). The α-amylase activity is directly related to the metabolic activity, leading to higher vigor of the rice

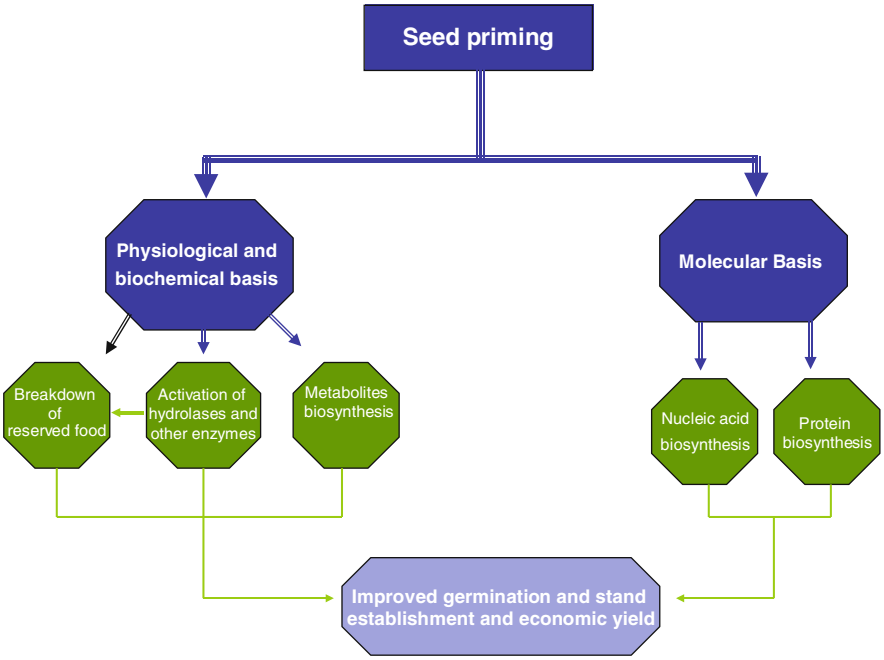

Fig. 2 Mechanism of rice seed priming. Improvement in germination, stand establishment, and economic yield owing to seed priming can be explained on a physiological, biochemical, and molecular basis. Seed-priming techniques increase the activity of hydrolases and some other enzymes (including antioxidants under stress conditions), which enhance the breakdown of reserve food. Meanwhile, some other metabolites are also synthesized. Nucleic acids and protein biosynthesis are also enhanced by seed priming techniques

seeds (Basra et al., 2005b; Farooq et al., 2006 k). Significantly higher and more rapid germination of osmoprimed rice seeds under low temperature (5°C) and salt (0.58% NaCl) stresses were observed. However, no significant changes in the activities of seed α-amylase and root system dehydrogenase were observed, while activities of seed β-amylase and shoot catalase were enhanced in low temperatures (He et al., 2002). Under salt stress, a significant increase in the activity of seed α-amylase, β-amylase, and root system dehydrogenase, and a moderate rise in the activity of shoot catalase occurred (He et al., 2002). Lee and Kim (2000), while investigating the effects of osmoconditioning and hardening on the germination of normal and naturally aged seeds, showed that the α-amylase activity of normal seeds was greater than the aged ones; the latter being more effective than the former. The α-amylase activity was positively correlated with the total sugars and germination rate. The increase in gibberellic acid concentration and the duration of exposure increased the α-amylase activity and seed germination (Vieira et al., 2002).

Lanthanum ion has been found to be effective in modulating the activities of seed enzymes in rice. The influence of soaking in lanthanum salt on the germination and seedling growth of rice indicated that the range of 1–20 mg L^{-1} increased

Table 5 Effect of various seed priming treatments on some metabolic changes in rice seeds

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Soluble Sugars				
Hardening 24 h	Coarse	KS-282	9.18 mg g^{-1} fresh weight	Basra et al. (2006b)
Hardening 24 h	Fine	Basmati-385	7.88 mg g^{-1} fresh weight	Basra et al. (2005b)
Hydropriming 48 h	Coarse	KS-282	2.11 mg g^{-1} fresh weight	Farooq et al. (2006c)
Ascorbate priming	Coarse	KS-282	4.53 mg g^{-1} fresh weight	Farooq et al. (2006c)
Osmohardening KCl	Coarse	KS-282	9.21 mg g^{-1} fresh weight	Farooq et al. (2006c)
Osmohardening $CaCl_2$	Coarse	KS-282	6.94 mg g^{-1} fresh weight	Farooq et al. (2006c)
Hardening 24 h	Coarse	KS-282	6.25 mg g^{-1} fresh weight	Farooq et al. (2006c)
Hydropriming 48 h	Fine	Super-Basmati	3.23 mg g^{-1} fresh weight	Farooq et al. (2006l)
Ascorbate priming	Fine	Super-Basmati	2.11 mg g^{-1} fresh weight	Farooq et al. (2006l)
Osmohardening KCl	Fine	Super-Basmati	4.0 mg g^{-1} fresh weight	Farooq et al. (2006l)
Osmohardening $CaCl_2$	Fine	Super-Basmati	6.56 mg g^{-1} fresh weight	Farooq et al. (2006l)
Hardening 24 h	Fine	Super-Basmati	4.97 mg g^{-1} fresh weight	Farooq et al. (2006l)
Reducing sugars				
Hydropriming 48 h	Coarse	KS-282	0.26 mg g^{-1} fresh weight	Farooq et al. (2006k)
Ascorbate priming	Coarse	KS-282	0.36 mg g^{-1} fresh weight	Farooq et al. (2006k)
Osmohardening KCl	Coarse	KS-282	0.44 mg g^{-1} fresh weight	Farooq et al. (2006k)
Osmohardening $CaCl_2$	Coarse	KS-282	0.40 mg g^{-1} fresh weight	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	0.30 mg g^{-1} fresh weight	Farooq et al. (2006k)
α-amylase activity				
Hydropriming 48 h	Coarse	KS-282	5.31 units*	Farooq et al. (2006k)
Ascorbate priming	Coarse	KS-282	5.78 units	Farooq et al. (2006k)

Table 5 (continued)

Seed priming treatment	Rice type	Variety/cultivar/genotype	Improvement recorded over control	Reference
Osmohardening KCl	Coarse	KS-282	6.60 units	Farooq et al. (2006k)
Osmohardening $CaCl_2$	Coarse	KS-282	5.80 units	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	5.4 units	Farooq et al. (2006k)
Hardening 24 h	Coarse	KS-282	6.60 units	Basra et al. (2006b)
Hardening 24 h	Fine	Basmati-385	8.05 units	Basra et al. (2005b)
Hydropriming 48 h	Coarse	KS-282	4.20 units	Farooq et al. (2006c)
Ascorbate priming	Coarse	KS-282	4.10 units	Farooq et al. (2006c)
Osmohardening KCl	Coarse	KS-282	6.40 units	Farooq et al. (2006c)
Osmohardening $CaCl_2$	Coarse	KS-282	5.30 units	Farooq et al. (2006c)
Hardening 24 h	Coarse	KS-282	4.31 units	Farooq et al. (2006c)
Hydropriming 48 h	Fine	Super-Basmati	4.00 units	Farooq et al. (2006l)
Ascorbate priming	Fine	Super-Basmati	3.56 units	Farooq et al. (2006l)
Osmohardening KCl	Fine	Super-Basmati	4.25 units	Farooq et al. (2006l)
Osmohardening $CaCl_2$	Fine	Super-Basmati	5.52 units	Farooq et al. (2006l)
Hardening 24 h	Fine	Super-Basmati	3.94 units	Farooq et al. (2006l)

* One unit of the enzyme's activity is the amount of enzyme that released 1 μmol of maltose by 1 mL original enzyme solution in 1 minute.

the vigor and proteinase, amylase, and lipase activities of seeds (Zhang et al. 2005). Promotion of germination in a highly dormant rice cultivar, Urucuia, was primarily related to an increase in α-amylase activity (Vieira et al., 2002). Likewise, indole acetic acid-soaked germinating rice seeds showed greater stimulation of α-amylase activity than gibberellic acid (Kim et al., 2006). In another study too, lanthanum nitrate enhanced the activities of α-amylase, proteinase, lipase, and other hydrolytic enzymes, and the contents of plant hormones such as indole acetic acid, gibberellic acid, and cytokinin, but abscisic acid contents changed a little (Fashui et al., 2003). Aged rice seed treated with lanthanum nitrate enhanced the respiratory rate and activities of superoxide dismutase, catalase, and peroxidase, and declined O_2^- contents and plasma membrane permeability (Fashui, 2002). Rice seed treatment with increased concentrations of gibberellic acid enhanced the activities of superoxide dismutase and catalase by 37.9 and 22.8%, respectively (Deshpande et al., 2003).

4.1.2 Metabolites

Carbohydrates constitute the major storage compounds in rice seeds. They are stored in the form of starch, which can not be consumed directly by the growing embryo. By the action of hydrolases, starch is converted into soluble sugars. Higher contents of soluble sugars are thus directly responsible for improved seed performance. In this context, the increased contents of total and reducing sugars and reduced nonreducing sugars in osmoprimed and hardened rice seeds are directly related to the activities of sugar hydrolyzing enzymes, as well as germination and seedling vigor (Table 5; Lee and Kim, 2000; Basra et al., 2005b, 2006b). Lee and Kim (1999) reported that optimum osmoconditioning of rice seed results in the disintegration of larger starch grains into tiny ones with the production of small holes in the starch granules and cavity between the embryo and endosperm.

Higher levels of sucrose and lower levels of total soluble sugars and fructose were observed in the primed hybrid rice seeds. Significant negative correlations between the fructose content in primed seeds and the fructose and total soluble sugars contents in stressed seedlings were also found. Priming decreased fructose, and increased free proline contents in seeds and fructose content in seedlings, and thus improved the salt tolerance of seedlings (Ruan et al., 2003). Hormonal priming of seeds also increases the level of soluble sugars in the rice grains. Increased contents of soluble sugars were recorded in cytokinin (Deshpande et al., 2003), gibberellic acid, and to a greater extent in indole acetic acid-treated rice seeds (Kim et al., 2006).

Osmolytes, including amino acids and derivatives, polyols and sugars, methylamines, and tertiary and quaternary ammonium compounds, are small low molecular weight organic solutes that are nontoxic and capable of maintaining cell and tissue water balance. All known osmolytes are compatible, do not perturb macromolecules, and more importantly stabilize membranes. Little work has been done on the changes in osmolyte levels in primed rice seeds. Reports show that a higher level of proline was observed in the primed seeds than in the control seeds, which also improved salt tolerance in hybrid rice seedlings (Ruan et al., 2003). In another

study, Deshpande et al. (2003) recorded a 24.5% increase in free proline content over the control group in naphthalene acetic acid primed hybrid rice seeds.

In nutshell, the production of sugars that can metabolize constitutes an important consequence of rice seed priming. These sugars provide a ready source of energy for the earlier production and establishment of seedlings. A build-up in the levels of free proline appears to be an important strategy, especially under suboptimal conditions. However, further studies are imperative on this aspect of seed priming in rice.

4.2 Molecular Basis

Studies associated with protein and nucleic acid synthesis fail to discriminate between the physiological events occurring during priming and those consequent to germination (McDonald, 2000). Therefore, it is important that due consideration be given to the understanding of the molecular basis of seed priming in terms of functional genomics (transcriptomics, proteomics, and metabolomics). Protein expression, taking place after the transcript synthesis, is commonly used by proteomics researchers to denote the presence or abundance of one or more proteins in a particular cell or tissue. Such reports are virtually lacking in the case of rice seed priming, although quite a few studies are available in other plant species (Gallardo et al., 2001; Wahid et al., 2008). Anuradha and Rao (2001) reported that the improvement of salinity tolerance in rice by brassino steroid seed treatments was associated with enhanced levels of nucleic acids and soluble proteins.

5 Seed Priming and Dormancy Management

The dormancy of seed results in arrested germination and various priming treatments have proven their worth in overcoming this physiological phenomenon. The dormancy breakdown can be accomplished by priming with salts, hormones, or other substances. Seed-germination tests of 18 accessions representing 16 rice species were conducted under a series of dormancy-breaking treatments, including hull removal, use of salts, hydrogen peroxide, and temperature regimes. These data revealed that (1) removal of the seed hull was extremely effective for breaking seed dormancy; (2) species responded differently to various temperature regimes, and no single regime was consistently effective in breaking seed dormancy in all species (although heat treatment generally promoted germination of the species); and (3) some species responded to certain chemical treatments effectively under the optimum temperature regimes. An appropriate combination of seed-hull removal, dry heat, or chemical treatments, and germination under the optimum temperature regimes for individual rice species provided the best results for breaking seed dormancy (Naredo et al., 1998).

Among the plant hormones, gibberellins are well-known for breaking the dormancy of dormant seeds and improving the germination of recalcitrant seeds

(Srivastava, 2002). Evaluation of gibberellic acid in breaking the seed dormancy of the highly dormant rice cultivar, Urucuia, subjected to predrying in a forced air circulation chamber (40°C) for seven days or soaked in 60 mg gibberellic acid L^{-1} concentrations at 30°C for 2, 24, or 36 h, revealed that all the treatments significantly reduced the dormancy of seed, which was tightly linked to an increase in α-amylase activity and seed germination. This further suggested that α-amylase activity is an efficient marker to study the seed dormancy in rice (Vieira et al., 2002).

In essence, the seed dormancy in rice can be broken by employing a wide range of seed treatments. However, priming with gibberellic acid, inorganic salts (particularly KNO_3), and thermal treatments, are more effective. Further work utilizing novel growth-promoting substances is imperative in rice.

6 Rice Seed Priming and Stress Tolerance

As such, rice carries an odd portfolio of tolerances and susceptibilities to stresses compared to other crops. It thrives in waterlogged soil and can tolerate submergence at levels that would kill other crops. It is moderately tolerant of salinity and soil acidity, but highly sensitive to drought and cold even where rice response to stress is superior to other crops. However, many rice-growing environments demand still greater tolerance than is found in the improved germplasm (Lafitte et al., 2004). Like other crops, rice is affected by various environmental constraints. Available literature on these lines is reviewed below:

6.1 Drought

Drought is generally avoided in irrigated rice-production systems, but it is a consistent feature across much of the 63.5 Mha of rainfed rice sown annually, most of which is in tropical Asia, Africa, and Latin America (Narciso and Hossain, 2002). In the newly introduced aerobic rice culture, the frequency and intensity of drought may increase manifold. Du and Tuong (2002), while testing the effectiveness of different osmotica to improve the performance of direct-seeded rice, noted that osmopriming with 14% potassium chloride solution and saturated calcium phosphate solution was successful in improving the seedling emergence, stand establishment, and yield under water-deficit conditions. Harris et al. (2002) reported that in drought-prone areas, primed rice seeds germinated well and seedlings emerged faster and more uniformly leading to increased yield. A germination trial of 11 varieties of upland rice under limited water conditions revealed early and synchronized emergence owing to seed priming (Harris and Jones, 1997).

In summary, the priming of rice seeds might be a useful way for better seedling establishment under water-limited soil conditions (Lee et al., 1998a).

6.2 Salinity

Salt stress is a major debacle to cereal production worldwide. Rice is a salt-sensitive crop, but at the same time it is the only cereal that has been recommended as a desalinization crop because of its ability to grow well under flooded conditions. This is because the standing water in rice fields can help leach the salts from the topsoil to a level low enough for subsequent crops (Bhumbla and Abrol, 1978). Despite its high sensitivity to salinity, considerable variation in tolerance was observed in rice (Akbar et al., 1972; Flowers and Yeo, 1981). Various strategies can be used to minimize the effect of salinity on the germination of rice seed and emerging seedlings. For instance, addition of putrescine (0.01 mM) to NaCl solution (150 mM) can reduce net accumulation of sodium and chloride ions in seeds and increase water uptake. This suggests that putrescine can alleviate the adverse effects of sodium chloride salinity during the germination and early seedling growth of rice (Prakash and Prathapasenan, 1988). In their study, Kim et al. (2006) reported that although gibberellic acid and indole acetic acid improved the salt tolerance of dehulled rice seeds, indole acetic acid was more effective. Brassinosteroids can also be used to induce stress tolerance. For instance, rice-seed treatment with brassinosteroids can reverse the inhibitory effect of salinity on germination and seedling growth (Anuradha and Rao, 2001). Osmopriming with mixed salts also improved the salinity tolerance in rice (He et al., 2002; Ruan et al., 2003). The above information suggests that improvement for salt tolerance is feasible in rice by a variety of priming techniques.

6.3 Low Temperature

The prevalence of low temperature at sowing results in poor rice-seed germination, seedling establishment, and vigor in several temperate rice-growing countries. Various strategies can be adopted to overcome the adversary of low temperature. For example, in a laboratory experiment, soaking rice seed in various concentrations of proline, betaine, putrescine, spermidine, and spermine increased germination and vigor at low temperatures. These compounds increased shoot growth by about 9 to 27% compared to growing the seedlings in water alone. Furthermore, the most effective concentrations to obtain an increase in shoot growth were 0.5, 2, 0.5, 0.05, and 0.05 mM for proline, betaine, putrescine, spermidine, and spermine, respectively (Naidu and Williams, 2004). Sasaki et al. (2005) noted that growth was promoted by hydrogen peroxide treatment under low temperature in a greenhouse. Soaking rice seeds in various combinations of indole acetic acid and glycinebetaine was also effective in inducing low-temperature tolerance in rice. Furthermore, the combined application of both was more effective in rice seed performance than their singular effects (Chen et al., 2005). In another study, sand priming improved the cold tolerance in direct-seeded rice (Zhang et al., 2006). He et al. (2002) also reported significantly higher and faster germination of osmoprimed rice seeds under

low-temperature (5°C) stress. This suggested that application of osmoprotectants is more effective in inducing low-temperature tolerance during seed germination.

6.4 Submergence and Water Logging

Excess water is a common constraint throughout the rain-fed rice production areas, such as South Asia and Southeast Asia and tropical Africa. Out of 40 Mha in Asia grown under rain-fed lowlands, about 15 Mha are frequently damaged by submergence (Huke and Huke, 1997). Submergence stress can also damage crops in irrigated areas due to high rainfall and/or impeded drainage, particularly early in the season. The annual average yield loss from submergence is estimated at about 80 kg ha^{-1} (Dey and Upadhaya, 1996). Although rice is adaptable to waterlogged conditions due to its capacity to develop aerenchyma, complete submergence can be lethal. Ruan et al. (2002b) reported improved seedling vigor index, seedling emergence, and stand establishment in flooded soil by osmopriming with calcium and sodium salts. Another study reported that rice-seed treatment with hydrogen peroxide can be used effectively to improve the submergence and flooding tolerance (Sasaki et al., 2005).

To sum up, seed priming with inorganic salts, polyamines, osmoprotectants, plant growth regulators, and hydrogen peroxide in optimum concentrations can be effectively used to improve tolerance against different stresses, including salinity, low temperature, and waterlogging/submergence. However, to accustom the direct seedling rice without puddling, a better understanding of the phenomena involved in drought and high-temperature tolerance at seedling development are imperative.

7 Conclusion

Seed invigoration tools have a great potential to improve the emergence and stand establishment under a wide range of field conditions. Among various techniques, osmopriming, osmohardening, hormonal priming and use of highly soluble and low molecular weight chemicals are of special consideration. These techniques can be effectively employed to enhance the crop performance under saline, submerged, and drought conditions. Seed invigoration techniques can also be employed to enhance the rice performance in direct-seeded cultures. There is a great variation in rice species, varieties/genotypes/hybrids and rice types regarding their responses to various priming treatments, which suggests that a lot of work still has to be done regarding the specific behavior of the rice material. Therefore, more precise invigoration techniques should be developed, using a range of salts, plant growth regulators, jasmonates, and osmolytes at varying concentration and for different durations. Optimal water potential, temperature range, and requirement for oxygenation should also be investigated. More research should focus on the use of commercial fertilizers as priming and seed-coating agents. Performance of invigorated seeds should be evaluated under a wide range of field conditions. These strategies should be employed

to improve tolerance against biotic and abiotic stresses. Thermal treatments with alternate cycles of low and high temperature should be studied in detail. Prolonged storage of primed and hardened seeds may be another critical factor in the technology transfer and marketing of primed rice and other crops' seeds. Therefore, more work should also be done to study the storage potential of primed seeds.

Studies on the possibility of integration of different invigoration tools should be done. Mechanisms of rice seed priming, particularly related to enzymatic activities, should be revealed. Moreover, the regulation of α-amylase by calcium and potassium ions during the priming may need to be investigated. Stress tolerance during germination is important. Thus, the pathway of anti-oxidant biosynthesis should be investigated. Studies on functional genomics of seed priming may pay rich dividends. Although baseline information is available, functional analyses of individual genes and proteins associated with the improved performances in seed priming are becoming increasingly important. The regulation/expression of genes/proteins involved is highly imperative to assessing the seed priming-based invigoration.

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Soil Management for Sustainable Crop Disease Control: A Review

R. Ghorbani, S. Wilcockson, A. Koocheki and C. Leifert

Abstract Excessive use of agrochemicals in conventional crop management has caused serious environmental and health problems, including loss of biodiversity and human disorders. A number of chemical biocides show complex chronic effects, such as changes in endocrine functions and immune systems. Application of different chemical biocides to the soil and plants has increased substantially over the last five decades. Total consumption of chemical fertilizers worldwide increased 10-fold from 1950 to 2000. This is also true for chemical biocides, with our annual current use of 3 billion liters and a value of 30 billion dollars. There is ample evidence that indicates that plants grown in rich soil associated with N-P-K availability are prone to pests and diseases. Managing and exploiting soil environmental conditions as part of an integrated control strategy can make a significant contribution to agricultural sustainability and environmental quality. Application of organic matters and practices that increase the total microbial activity in the soil might enhance the general suppression of pathogens by increasing competition for nutrients. The choice of crops in a rotation with plants less susceptible to specific pathogens causes a decline in population due to natural mortality and the antagonistic activities of coexistent root zone microorganisms. Plants growing in disease-suppressive soil resist diseases much better than in soils with low biological diversity. Understanding the effect of soil's environmental factors on plant disease incidence and the best crop management strategies to prevent, avoid, escape, and control diseases were the aims of this literature review. This article, which comprises the main topics on soil fertility associated with N-P-K and other macro- and micronutrients, and also soil pH, structure and texture, organic matter, and microbial reserves, describes the use of various crop management practices that reduce the incidence of plant diseases.

Keywords Biodiversity · Compost · Cropping system · Diseases · Organic farming · Organic matter · Microbial biomass · Nitrate · Soil fertility · Sustainability

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1 Introduction

Plant diseases create challenging problems and pose real economic threats in agricultural ecosystems. It has been evidenced that despite a wide use of chemical biocides in crop production, the losses due to pests and diseases are still significant. In this respect, crops lost due to weeds on a global basis have been estimated to be 10%, and the world market for chemical biocides is about U.S.$30 billion annually (Marshall et al., 2003). Plant diseases occur when a susceptible host and a disease-causing pathogen meet in a favorable environment (Sullivan, 2001). If any one of these three conditions is not met, there will be no disease. Many disease management practices, such as the use of fungicides and fumigants, focus on controlling pathogens when disease symptoms are apparent, which is often too late to be effective. A more reliable approach is to concentrate on the period before disease infection occurs and encourage conditions that are unfavorable for the pathogen and favorable for the plant. This article emphasizes the need to make the soil environment less favorable for the pathogens, and thus make host plants less susceptible to diseases.

Soil is the fundamental medium for crop growth in all production systems. The success of any system depends, to a large extent, on the soil characteristics such as the nutrient supply and structural characteristics that affect rooting. However, soil conditions for plant growth can influence the occurrence and severity of plant diseases. Managing and exploiting the suppressive effects of the soil environment as part of an integrated control strategy can make a significant contribution to agricultural sustainability and environmental quality (Quimby et al., 2002). A soil is considered to be suppressive against plant diseases if pathogens cannot become established, or can become established but do not cause disease, or become

established and produce disease for a short time but then decline. The degree of suppression is linked to soil physical conditions, fertility level, biodiversity and populations of soil organisms, and soil management (Sullivan 2001). The soil environment influences crop growth indirectly by affecting weed growth, pests, and diseases, as well as by directly supplying water and nutrients. However, while the general principles are theorized, there is a lack of detailed knowledge about soil factors and soil environmental conditions that influence the severity of plant diseases. This is essential to facilitate plant disease management. The goal of this review paper was to find out the best management strategies for sustainable crop disease management without using agrochemicals.

2 Soil Fertility

Successful colonization of plants by pathogens requires efficient utilization of nutrient resources available in host tissues (Snoeijers et al., 2000). Excessive fertilizer applications can increase a plant's susceptibility to diseases if they cause prolific growth of foliar and other parts (Davies et al., 1997). Researchers have found conflicting results in the effects of soil fertility on disease development in different plants and pathogens. Portela et al. (1999) found that the recovery of chestnut (*Castanea dentata*) from ink disease (*Phytophthora cinnamomi*) is poor where there are restrictions to root expansion caused by poor soil fertility, low aeration, and high-soil compaction resulting from infrequent soil disturbance by tillage. Maynard et al. (2007) reported a relationship between cavity spot (*Pythium* spp.) in carrots and low levels of calcium in their roots and petioles. Later, it was found that high levels of soil potassium may lead to a build-up of potassium in the plants that affects calcium uptake and hence the development of cavity spot (Hiltunen and White, 2002).

2.1 Nitrogen

The most commonly studied nutrient element in soil in relation to plant diseases is nitrogen. Abundant nitrogen encourages succulent growth, a prolonged vegetative period, and delayed maturity of the plant, which increases the period of susceptibility to pathogens. Deficient plants are weaker and slower growing, and are also more susceptible to pathogens (Agrios, 1997). Separating the direct and indirect effects of a nitrogen supply on the host-pathogen interaction is difficult because their influence on the dynamics of the pathogen via crop growth, crop physiology, and crop microclimate is confounded (Sasseville and Mills, 1979). Direct changes in host susceptibility to infection with higher levels of nitrogen have been postulated but are still controversial (Savary et al., 1995). Growth and disease responses to high levels of nitrogen have been documented for a range of plants and pathogens (Marti and Mills, 1991; Sasseville and Mills, 1979; Smiley and Cook, 1973). The effect of the soil nitrogen level on disease development in different agricultural crops is shown in Table 1. Similar results were observed in noncultivated plant species. For example,

Table 1 Effect of change in N-P-K levels on plant disease development

Host	Pathogen	Change in		Reference
		Nutrient	Disease	
Nitrogen:				
Pear	*Erwinia Amylovora*	+	+	Agrios (1997)
Wheat	*Puccinia graminis*	+	+	Agrios (1997)
	Erysiphe graminis	+	+	Agrios (1997)
	Rhizoctonia solani	+	+	Colbach et al. (1996)
	Gaeumannomyces graminis	+	+	
Rice	*Rhizoctonia solani*	+	+	Cu et al. (1996)
Sugar beet	*Sclerotium rolfsii*	–	+	Agrios (1997)
Potato	*Alternaria solani*	–	+	Rosen and Miller (2001)
Tomato	*Fusarium oxysporum*	–	+	Agrios (1997)
Phosphorus:				
Wheat	*Septoria*	+	+	Agrios (1997)
Barley	*Gaeumannomyces graminis*	+	–	Agrios (1997)
Potato	*Streptomyces scabies*	+	–	Agrios (1997)
Cotton	*Fusarium oxysporum*	+	+	Jones et al. (1989)
Spinach	*Cucumber mosaic virus*	+	+	Agrios (1997)
Cauliflower	*Rhizoctonia solani*	+	–	Chauhan et al. (2000a)
Linseed	*Fusarium oxysporum*	+	+	Singh (1999)
Cowpea	*Colletotrichum lindemuthianum*	–	+	Adebitan (1996)
Potassium:				
Wheat	*Puccinia triticina*	+	–	Sweeney et al. (2000)
	Puccinia graminis	+	–	Agrios (1997)
Soybean	*Phytophthora sojae*	+	+	Pacumbaba et al. (1997)
	Soybean mosaic virus (SMV)	+	+	Pacumbaba et al. (1997)
Rice	*Magnaporthe grisea*	+	+	Agrios (1997)
	Meloidogyne incognita	+	+	Agrios (1997)
Onion	*Peronospora destructor*	+	–	Develash and Sugha (1997)
Cotton	*Verticillium dahliae*	–	+	Mucharromah and Kuc (1991)
Tomato	*Alternaria solani*	+	–	Agrios (1997)

+ Indicates an increase in one factor will either increase (+) or decrease (–) the other factor. For example, in the case of wheat, an increase in nitrogen (+) in most cases has increased (+) and only in one case has the reverse been observed.

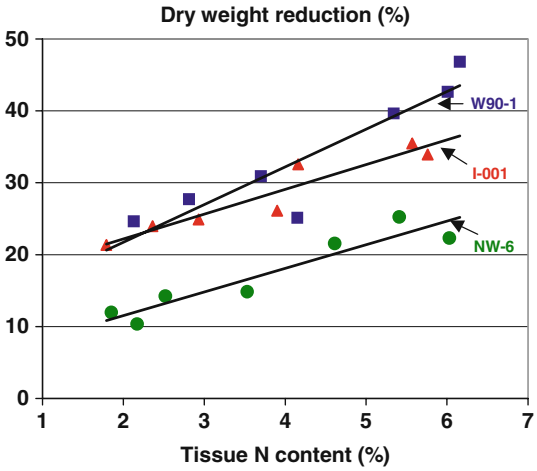

Fig. 1 The effect of plant tissue-nitrogen content on *Ascochyta*-induced dry weight reduction of common lambs quarters treated with 500 l ha^{-1} suspension containing 10^7 spores ml^{-1} of different *A. caulina* isolates. Each point represents the mean of eight replications at 10 days after spraying

Ghorbani et al. (2002) found that dry weight reduction due to disease development caused by *Ascochyta caulina* in the common weed species *Chenopodium album* increased with increasing plant-tissue nitrogen (Fig. 1). Apart from the possible effects of nitrogen on shoot architecture and the microclimate (humidity) affecting germination of the pathogen, increased nitrogen may affect cuticular properties, cell-wall structure and the metabolic activity of leaves, and hence disease susceptibility (Snoeijers et al., 2000). A nitrogen-limiting environment may also be one of the cues leading to disease symptom development after infection by pathogens in some plant species.

In addition to the amount of nitrogen available to the host or pathogen, the form of ammonium or nitrate may also influence plant disease incidence. The ammoniacal (NH_4^+) form of nitrogen reduced the severity of the *Phymatotrichum omnivorum* in cotton, while the nitrate (NO_3^-) form increased plant mortalities (Matocha and Vacek, 1997). Nitrate forms of nitrogen fertilizer suppressed the *Fusarium* wilt of tomato, while the ammonia form increased disease severity (Woltz and Jones, 1973). The disease severity caused by *Fusarium* spp., *Plasmodiophora brassica*, *Sclerotium rolfsii*, and *Pyrenochaeta lycopersici* increased when an ammonium fertilizer was applied (Agrios 1997).

The effect of the form of nitrogen could be due to soil pH. Ammonium ions (NH_4^+) are absorbed by the roots through exchange with H^+ ions that are released to the surrounding medium, thus decreasing soil pH (Agrios, 1997). The nitrate form tends to make the root zone less acidic. Basically, the beneficial effects of high pH are lost by using acidifying ammonium nitrogen (Sullivan, 2001). Thus, the use of ammonium fertilizer, such as ammonium sulphate, will decrease soil pH and encourage diseases that are favored by low pH. With nitrate fertilizers, on the other hand, those diseases that are favored by neutral to alkaline pHs will be more severe. For example, studies using nitrate and ammonium forms of nitrogen against the *Fusarium* wilt of tomato have shown that application of nitrate to soil with an already

high pH improves wilt control (Woltz and Jones, 1973). Although a wide range of interactions of pathogens and their hosts are involved, as Huber and Watson (1974) concluded, it is generally the form of nitrogen available to the host or pathogen that affects disease severity or resistance, rather than the amount of nitrogen. Nitrogen is assimilated by plants in both ammonium and nitrate forms, together with very small amounts of organically bound nitrogen. The ammonium form is rapidly converted to amino acids, whereas nitrate can be stored. It is the nitrate form that forms the main source of nutrients for pathogens (Lampkin, 1999).

2.2 Phosphorus

Phosphate (PO_4) plays a central role as a reactant and effector molecule in plant-cell metabolism. However, phosphate is the least accessible macronutrient in many ecosystems, and its low availability often limits plant growth (Abel et al., 2002). Soil phosphate can also be critical for disease development (Sullivan, 2001). Phosphites (H_2PO_3) are alkali metal salts of phosphoric acid ($HPO(OH)_2$) that are being widely marketed either as agricultural fungicides or as superior sources of phosphorus for plant nutrition. Published research conclusively indicates that phosphite functions as an effective control agent for a number of crop diseases caused by various species of pathogenic fungi belonging to the genus *Phytophthora* (McDonald et al., 2001). There are many reports of an association between the level of available soil phosphorous and crop disease development (Table 1). The optimum level for individual crops will vary from soil to soil and disease to disease, but should be determined. Subsequent careful monitoring and management of available phosphorus and its balance with other nutrients could then be considered in an overall strategy for crop-disease management.

2.3 Potassium

Potassium fertility is also associated with disease development (Table 1). For example, spraying aqueous solutions (20 or 50 mM) of potassium oxalate, potassium phosphate dibasic (K_2HPO_4), or tribasic (K_3PO_4) on cucumber plants (*Cucumis sativum*) induced systemic resistance to *Collectorichum lagenarium*, *Cladosporium cucumerium*, *Dydimella bryoniae*, *Sphaerotheca fuliginea*, *Pseudomonas lachrymans*, *Erwinia tracheiphila*, tobacco necrosis virus (TNV), and cucumber mosaic virus (CMV) (Mucharromah and Kuc, 1991). The decrease in the severity of black spot disease (*Alternaria brassicae*) in field mustard (*Brassica campestris*) by applying potassium was due to increased production of phenolics in plants, which inhibit conidial germination and decrease sporulation of *A. brassicae* (Sharma and Kolte, 1994). The rate and form of potassium and its balance with other soil nutrients are important to consider in crop disease management and the optimum balance for a range of situations needs to be determined.

2.4 Other Macro- and Micro-Nutrients

Studies with other elements such as calcium, magnesium, iron, zinc, and other micronutrients indicated similar relationships between their levels in the soil and the susceptibility or resistance to certain diseases. Calcium had a great effect on controlling *Pythium* damping off in wheat, sugarbeet, soybeans, peanut, peas, peppers, beans, tomatoes, and onions (Wen-Hsiung and Ching-Wen, 1989). The (Ca+Mg)/K ratio appears to play a role in several crops—for example, *Zea mays, Cucumis melo*, *Brassica juncea*, *Brassica napus*, *Vigna sinensis*, *Solanum esculentum*—as well as root-knot nematode damage caused by *Meloidogyne incognita* (Bains et al., 1984). The severity of panicle blast (*Pyricularia grisea*) in four genotypes of rice was related to nutrient concentrations in panicle tissues. Nitrogen, phosphorus, and magnesium contents in panicle tissue were positively correlated, whereas potassium and calcium were negatively correlated with panicle blast severity in rice. The low disease severities in the improved cultivar Guarani were associated with high K and Zn, and low N, P, and Mg tissue concentrations (Filippi and Prabhu, 1998). Matocha and Hopper (1995) and Matocha and Vacek (1997) indicated an association between levels of iron deficiency chlorosis (and possibly Zn) and the severity of *Phymatotrichum omnivorum* incidence on cotton. Analyses of soil samples proved that at least two plant nutrients, Fe and Mg (and possibly Zn and Ni), were usually in short supply where this pathogen reached severe proportions. Chloride fertilization suppressed powdery mildew (*Erysiphe graminis*) and leaf rust (*Puccinia recondita*) in wheat plants (Engel et al., 1994). Duffy et al. (1997) showed that biocontrol activity of *Trichoderma koningii* for controlling take-all disease (*Gaeumannomyces graminis* var. *tritici*) in wheat was positively correlated with iron, nitrate-nitrogen, boron, copper, soluble magnesium, and percent clay in the soil, but negatively correlated with soil pH and available phosphorus. Lee et al. (1998) reported that silicon soil amendments reduced the disease severity of blast (*Magnaporthe grisea*) in rice growing on several soils that were deficient in silicon.

2.5 Soil Organic Matter

Soil organic matter, soil microbes, and pesticides impact the growth and development of plant pathogens either by supplying nutrients or by providing favorable or unfavorable environments for plants and pathogens (Newman, 1985). The role of organic matter in crop protection is associated with increased microbial activity, reduced aggressiveness and infestation of pathogens, increased viral resistance, and a reduction in soil tiredness or toxicity. The addition of organic matter improves the vigor of the plant as a result of physical and chemical improvement of the soil and also increases resistance of the individual plant as a result of the uptake of phenols, phenolic acids, and other compounds such as salicylic acid, which have an antibiotic effect and also work directly on pathogens (Lampkin, 1999). Applying organic manures makes a direct contribution to the antiphytopathogenic potential of soils.

This is particularly important in the case of fungal damping-off diseases such as *Rhizoctonia*, *Fusarium*, and *Pythium* (Lampkin 1999). Various chemicals in the soil are known to contribute to the antiphytopathogenic potential of the soil. The breakdown of organic manures results in the release of carbon dioxide, which is harmful to some pathogens in high concentrations. Toxins available in the crop residue, which is produced against other biological agents such as weeds and is called allelochemicals, may also act against plant pathogens (Lampkin, 1999). In most studies, adding mature organic matter such as compost to the soil induces disease resistance in many plants. Typically, the level of disease suppression was related to the level of total microbiological activity in a soil (Sullivan, 2001).

In many cases, the incorporation of organic matter in the soil showed beneficial alternatives to chemicals for plant disease control. For example, Viana et al. (2000) reported that mature cattle manure and sugarcane husks are efficient alternatives for control of bean damping-off (*Sclerotinia sclerotiorum*). Farmyard manure applied at 5 t/ha, once every three years, reduced dry root rot (*Macrophomina phaseolina*) to 32% in groundnut (*Arachis hypogaea* L.) compared with untreated plants (Harinath and Subbarami, 1996). Ceuster and Hoitink (1999) reported that *Pythium* and *Phytophthora* root rots can be controlled most effectively in container media amended with composted bark. However, there are reports that using organic fertilizers increased disease development. For example, Chauhan et al. (2000a) found that increasing the application of farmyard manure from 25 to 75 t/ha increased the disease severity of stem rot (*Rhizoctonia solani*) in cauliflower (*Brassica oleracea*).

Application of compost may improve soil microorganism communities. As the active microbial biomass increases, the capacity to utilize carbon, nutrients, and energy in the soil is increased, and thus these resources will be very limited for the soil-borne pathogens (Sullivan, 2001). Compost acts as a food source and shelter for antagonists that compete with plant pathogens, organisms that prey on and parasitize pathogens, and beneficial microorganisms that produce antibiotics (Sullivan, 2001). Disease suppression in compost is very much related to the degree of decomposition; as the compost matures, it generally becomes more suppressive. However, readily available carbon compounds found in low-quality immature compost suppressed *Pythium* and *Rhizoctonia* (Nelson et al., 1994). The recommended approaches for increasing suppression of compost are: curing the compost for four months or more before using it, or incorporating the compost in the field soil several months before planting and inoculating it with specific biocontrol agents (Hoitink et al., 1997). Examples of beneficial organisms used to inoculate compost are strains of *Trichoderma* and *Flavobacterium*, added to suppress *Rhizoctonia solani* in potatoes. *Trichoderma harzianum* acts against a broad range of soil-borne fungal crop pathogens, including *R. solani*, by production of antifungal exudates (Sullivan, 2001).

In many studies, application of compost extracts or compost "teas," which are filtered mixtures of compost materials and water, showed promising crop protection effects depending on the length of the soaking period referred to as "extraction time." The mechanisms by which compost extracts work are not well-known, but seem to vary depending on the host/pathogen relationship and the mode of application (Weltzien, 1989). Goldstein (1998) reported that composts and compost

extracts activate disease resistance genes in plants. These are the genes that are normally activated in response to the presence of a pathogen. They mobilize chemical defenses against the pathogen invasion. Plants growing in compost may have these disease-prevention systems already activated (Sullivan, 2001).

The contribution of compost to nitrogen fertility must also be taken into account because nutrient effects may influence the severity of pathogens, as explained previously. *Phytophthora* die back of *Rhodododendron*, *Fusarium* wilt of cyclamen are examples of diseases that increase in severity as a result of excessive nitrogen fertility introduced into container media with composted biosolids (Ceuster and Hoitink, 1999). However, this effect may not occur when composts are produced from products with a high carbon-to-nitrogen ratio (C/N), such as wood residues. Most high C/N ratio composts (>70:1) immobilize nitrogen. Thus, plants grown in such products suffer from chronic nitrogen deficiency, resulting in a lack of growth and increased susceptibility to stress pathogens or insects (Ceuster and Hoitink, 1999). High C/N ratio tree bark compost may suppress *Fusarium* wilts, but with lower C/N ratio composts they may become more severe as a result of the excess nitrogen, which favors *Fusarium* (Hoitink et al., 1997). The moisture content following the peak heating stage of compost is critical to the range of organisms inhabiting the finished product. Compost with at least 40–50% moisture will be colonized by both bacteria and fungi and will be suppressed for *Pythium* disease (Hoitink et al., 1997).

Certain biocides can indirectly increase pathogen density in soil. As the roots of weeds or other plants treated with herbicides die, they become much more easily colonized by pathogens such as *Pythium, Rhizoctonia*, and *Fusarium* than live plants (Sullivan, 2001). This is because dead roots exude sugars and other carbon compounds that are food sources for pathogens. Applying glyphosate or paraquat in bean fields resulted in an increase in *Pythium* in the soil for 21 days after treatment (Descalzo et al., 1998).

Adding mature organic matter to the soil, therefore, improves plant health and induces disease resistance in many plants. However, as Ceuster and Hoitink (1999) suggested, many aspects of organic amendments, such as compost and organic manure quality, must be controlled to obtain consistent results because of their variable nature. To get more consistent results, the compost itself needs to be stable and of consistent quality. The composition of the organic matter from which the compost is prepared, the composting process, the stability or maturity of the finished product, the quantity of available plant nutrients it provides, and the time of application must all be carefully considered. To formulate an overall disease management strategy, farmers should know the C/N and N/P ratios in organic fertilizer before application. They should be able to carefully monitor the rate, form, balances, and availability of N-P-K.

3 Soil Microbial Biomass

Soil microbial biomass is composed of eukaryotes such as fungi, yeasts, protozoa and algae, and prokaryotes including eubacteria, actinomycetes, and archaea,

and microorganisms that vary from soil to soil (Shannon et al., 2002). They have a critical role in nutrient cycling as a relatively labile source of plant nutrients such as nitrogen, phosphorus, and sulphur, and in promoting soil aggregation (Shannon et al., 2002). One of the prime determinative factors of soil microbial status is, however, the type and amount of organic materials that enter the soil ecosystem. The vast majority of soil microorganisms are heterotrophic, with the exception of phototrophic algae and bacteria in surface soils, and chemolithotrophic prokaryotes, such as the nitrifying bacteria. They require organic materials as both carbon and energy sources (Shannon et al., 2002). Management practices that involve various inputs of organic materials into soils might therefore be predicted to modify soil microbial populations. In particular, the manipulation of the quality and quantity of organic inputs has implications for the activity of soil microorganisms, the soil food web, and the biological processes of nutrient transformation (Stockdale et al., 2002).

There are many reports that organisms in the soil might be antagonistic to plant pathogens. For example, over 100 species of fungi trap and prey on nematodes (Jatala, 1986), and many fungi are hyperparasites of other fungi (Adams, 1990). For example, species of *Trichoderma* that secrete lytic enzymes are active against fungal cell walls (Sivan and Chet, 1989); *Talaromyces flavus* is able to attack sclerotia of *Sclerotinia sclerotiorum*, and *Verticillium dahlae* (Mclaren et al., 1989) and *Sporidesmium sclerotiorum* are able to attack the sclerotia of five important plant pathogens (Adams, 1990). Sullivan (2001) reported a direct correlation between general microbial activity and an amount of microbial biomass, and the degree of *Pythium* suppression.

Nematodes and protozoans feed upon microbial populations and consequently affect organic matter decomposition. It is likely that such feeding ultimately liberates nutrients immobilized in microbial cells or reduces competition between microorganisms so that mineralization is actually accelerated. These activities not only influence the general nutrition, health, and vigor of higher plants (which affects disease susceptibility), but also determine the competitive behavior of root-infecting fungi and their microbial antagonists (Curl, 1988). Streptomycetes are common filamentous bacteria that are effective, persistent soil saprophytes and often associated with plant roots. They are well-known producers of antibiotics and extracellular hydrolytic enzymes. Samac et al. (2003) reported that *Streptomycetes* have the potential to contribute significantly to an integrated disease management system that includes alfalfa and other crops, such as potato, maize, and soybeans, due to their ability to colonize plants and decrease damage from a broad range of pathogens.

There are several factors that affect soil microorganisms' activities, such as soil moisture, temperature, soil organic matter, and agronomic practices such as irrigation and the use of chemicals (Hiltunen and White, 2002). Populations of soil microbes and plant pathogens are affected by field management policies and agricultural practices. The application of organic matter and all treatments that increase the total microbial activity in the soil might enhance general suppression of pathogens by increasing the competition for nutrients. The choice of crops in a rotation

with plants less susceptible to specific pathogens causes a decline in population due to natural mortality and the antagonistic activities of coexistent root-zone microorganisms (Fry, 1982).

Crop rotation may also change microbial population beyond that normally associated with pathogen host range and saprophytic survival (Peters et al., 2003). Rotation is the most successful in limiting the impact of biotrophic pathogens that require living host tissues, or those pathogens with low saprophytic survival capability (Bailey and Duczek, 1996). It is least successful in reducing disease caused by pathogens with a wide host range, or that produce long-lived survival structures, such as sclerotia or oospores (Umaerus et al., 1989). Seed source has also been shown to influence *Rhizoctonia* disease severity, presumably due to differing loads of microbial antagonists (Jager and Velvis, 1983). Mechanisms by which endophytes can act as biocontrol agents include production of antibiotic agents (Lambert et al., 1987), siderophore production (Kloepper et al., 1980), nutrient competition (Kloepper et al. 1980), niche exclusion (Cook and Baker, 1983), and induction of systemic acquired host resistance (Chen et al., 1995). Bacterial endophytes can thus play a role in pathogen suppression (Chen et al., 1993; Sturz et al., 1998, 2000), and complementary crop sequences can encourage beneficial allelopathy (Sturz et al., 1998). Mycorrhiza symbiotic systems (microbial associations with mycorrhizal fungi and bacteria that live on and near the roots) help the plant root systems access available nutrients. Mycorrhizal fungi also protect plant roots from diseases in several ways. These include the provision of a physical barrier to the invading pathogen, secretion of antagonistic chemicals, competition with the pathogen, an increase in the nutrient-uptake ability of plant roots, and changing the amount and type of plant root exudates (Sullivan, 2001).

4 Soil pH

Soil pH influences plant disease infection and development directly by effects on the soil-borne pathogen and microorganism populations, and indirectly through the availability of soil nutrients to the plant host. For example, sporangium formation, zoospore release, and the mortality of zoospores of *Phytophthora cinnamomi* was reduced by pH values less than 4.0 (Blaker and MacDonald, 1983).

The percentage of peanut stems infected by *Sclerotium rolfsii* was greater at soil pH 5.6 than in more alkaline soil, although the disease did develop at soil pHs of 8.7 and 9.8 (Shim and Starr, 1997). Holmes et al. (1998) studied the effect of pH ranging from 4.5 to 8.0 on the biocontrol of sugar beet damping-off (*Pythium ultimum*) using *Pythium oligandrum* as an antagonist. They showed that this species was able to control pre- and postemergence damping-off of sugar beet, but only at pH 7.0 and 7.5. Potato common scab (*Streptomyces scabies*) can be severe from pH 5.2 to 8.0 or above (Dominguez et al., 1996). Sullivan (2001) reported that this disease is more severe in soils with pH levels above 5.2, but is generally suppressed at lower pHs. Sulphur and ammonium sources of nitrogen acidify the soil and also reduce the

incidence and severity of potato scab. Liming, on the other hand, increases disease severity.

While lowering the pH is an effective strategy for control of potato scab, increasing soil pH or calcium levels may be beneficial for disease management in many other crops (Sullivan, 2001). Studies on the effect of soil characteristics on *Fusarium* wilts in banana plants indicated relationships between disease incidence and pH values, cation exchange capacity (CEC), sodium in solution, and iron (Dominguez et al., 1996). Blank and Murray (1998) reported that soil pH from 4.7 to 7.5 did not have a significant influence on the germination of *Cephalosporium gramineum* conidia.

Soil pH is important in soil fertility and nutrient availability. In some diseases, the weakening of the host through altered nutrition induced by soil acidity may affect the incidence and severity of the disease. For example, a more acid soil also fosters better uptake of manganese and adequate manganese-stimulated disease resistance in some plants (Sullivan, 2001). A direct correlation between adequate calcium levels, and/or higher pH, and decreasing levels of *Fusarium* occurrence has been established for a number of crops, such as tomato, cotton, and melons (Jones et al., 1989). Application of lime (calcium carbonate) increases soil pH and reduces the incidence of cavity spot (*Pythium* spp) in carrots (Hiltunen and White, 2002). Lime and gypsum (calcium sulphate) amendments could change soil acidity, nutrient availability, and diseases severity.

5 Soil Texture and Structure

Soil texture and structure could have effects on plant diseases because they affect water-holding capacity, nutrient status, and gas exchange, as well as root growth. For example, maximum stem rot (*Rhizoctonia solani*) incidence in cauliflower (*Brassica oleracea* var. *botrytis*) occurs more in sandy soils and less in clay soils (Chauhan et al., 2000b). Radial spread of the wheat root rot (*Rhizoctonia solani*) pathogen in the soil was twice as fast in sand as compared to loamy sand, which in turn was more than twice that in sandy clay loam soil (Gill et al. 2000). Bolanos and Belalcazar (2000) indicated that plants were more affected by *Erwinia chrysanthemi* in the sandiest soil. Reproduction of *Meloidogyne incognita* was greater in coarse-textured soils than in fine-textured soils, whereas *Rotylenchulus reniformis* reproduction was greatest in loamy sand. In this study, the population densities of *M. incognita* were inversely related to the percentage of silt and clay, but *R. reniformis* was favored by moderate levels of clay plus silt of about 28 wt% (Koenning et al., 1996).

Interaction between soil texture and tillage is important in many diseases. The severity of early season damping-off (*Phytophthora sojae*) was not affected by tillage, but there was an interaction between tillage and texture (Workneh et al., 1999). It was detected with greater frequency in conservation tillage (less soil ploughing and more crop mulch-and-cover) than in conventional tillage in silt loam and loam soils. However, in sandy loam, the frequency was significantly greater in

conventional tillage than in conservation tillage. Population densities of *Heterodera glycines* were significantly affected by both tillage and soil texture, but overall there was no tillage-texture interaction. An inverse relationship between population densities of *Heterodera glycines* nematodes and percent clay in no-till fields that were left undisturbed from harvest to planting was observed, but little or no change in nematode densities was shown with increasing clay content in tilled fields (Workneh et al., 1999).

Adverse soil structural conditions due to soil compaction or poor drainage greatly increase the chances of serious infection with many plant pathogens. In the case of wheat take-all (*Gaeumannomyces graminis*), a low level of disease was tolerated in heavy soil without much effect on yield. However, when compaction caused slower drainage, the same level of disease was much more harmful (Davies et al., 1997). Poor soil aeration caused by poor soil structure, soil type, or water-logging was associated with the development of cavity spot (*Pythium* spp.) disease in carrots (Hiltunen and White, 2002). The pea root rot complex (*Fusarium* spp.) is known to be affected by compaction, temperature, and the moisture of the soils. Chang (1994) showed that an increase in soil bulk density due to compaction significantly increased root rot incidence and disease severity, and drastically reduced the fresh weight of pea plants due to the disease. Tillage practices that reduce soil compaction, increase drainage, and increase soil temperature, have shown to generally reduce the severity and damage caused by root rot pathogens to many vegetables such as beans (Abawi and Widmer, 2000). Breaking hard pans and subsoiling after seedbed preparation were found to reduce *Fusarium* root rot damage in beans due to greater penetration and formation of roots (Burke et al., 1972). There are many reports that applying organic matter improves soil structure. Moreover, Forge et al. (2003) reported that the use of organic materials because mulches can have profound effects on the structure of the soil food web (feeding relationships between the plants and animals in the soil), which is relevant to the turnover of the microbial biomass and macronutrients.

6 Soil Moisture and Temperature

The amount and duration of moisture availability such as that in the form of precipitation or irrigation on the plant surfaces or around the roots, relative humidity in the air, and dew is of great importance for microbial life stages (Colhoun, 1973; Hoagland, 1990). In many diseases affecting the underground parts of plants such as roots, tubers, and young seedlings, the severity of the disease is proportional to the amount of soil moisture and is greatest near the saturation point. Such an example is *Pythium*, which causes damping-off of seedlings and seed decay (Hiltunen and White, 2002).

The increased moisture seems to affect the pathogen primarily, which multiplies and moves (zoospores in the case of *Pythium*) best in wet soils (Agrios, 1997). Increased moisture may also decrease the ability of the host to defend itself through

reduced availability of oxygen in water-logged soil, and by lowering the temperature of such soils. The cavity spot disease of carrots caused by the genus *Pythium* (e.g., *P. violae* and *P. sulcatum*) was more common on flat, badly drained fields, and those with poor soil structure than other soils (Hiltunen and White, 2002). High soil moisture levels promoted the development of *Phytophthora citrophthora* (root rot) of citrus (Feld et al., 1990). Many soil fungi (e.g., *Phytophthora*, *Rhizoctonia*, *Sclerotinia*), some bacteria (e.g., *Erwinia* and *Pseudomonas*), and most nematodes usually cause their most severe symptoms on plants when the soil is in field-capacity moisture but not flooded. Most bacterial diseases and also many fungal diseases of young tender tissues are particularly favored by high soil moisture or high relative humidity (Agrios, 1997).

Dry conditions are sometime unfavorable to the host and may predispose it to infection. For example, drought stress has long been known to enhance the invasion of groundnuts by *Aspergillus flavus* (Wotton and Strange, 1987). *Fusarium solani*, which causes dry rot in beans, and *Fusarium roseum*, which causes seedling blight, grow fairly well in rather dry environments, and can cause more severe diseases on water-stressed plants than where water is more freely available (Agrios, 1997). Irrigation should aim to maintain a water level that keeps a crop growing and no more. Farmers should carefully consider soil moisture content (Hiltunen and White, 2002) and monitor crop water usage and demand (Hulme et al., 2000).

Soil temperature does not appear to be as important as soil moisture in relation to plant diseases. Pathak and Srivastava (2001) reported that soil moisture and soil temperature had combined effects on the development of *Rhizoctonia bataticola* in sunflowers. In that study, with increasing soil moisture and decreasing soil temperature, disease development decreased. Root rot disease (*Rhizoctonia solani*) in wheat seedlings was more severe at a lower temperature of 10°C when soil moisture levels were optimum for plant growth, but under relatively dry conditions, disease levels were similar at all tested temperatures of 10°C, 15°C, 20°C, and 25°C (Gill et al., 2001). It has also been shown that a single solarization treatment significantly increased yields by +116%, and strongly reduced nematode infestation of −99% of infested plants and of −98% of the root gall index in the following melon crop. It

Fig. 2 Soil solarization for only two weeks controls weeds effectively

also suppressed annual weed emergence three years later (Candido et al., 2007). In a field experiment conducted in Mashhad University of Iran (Fig. 2), solarization for only two weeks in April 2006 controlled weeds significantly.

7 Cropping System and Agricultural Practices

Many researchers have suggested that increasing pest and disease pressure in agroecosystems is due to changes in cropping systems and agricultural practices since World War II, particularly with the application of agrochemicals (Altieri and Nicholls, 2003). Cropping systems and cultural practices such as rotation, fertilization, and application of pesticides can affect soil characteristics and disease development in plants. Two major differences between conventional and organic production systems lie in their approaches to soil fertility and pest management. Experiments and on-farm surveys conducted by Brown et al. (2000) have suggested that organic farm management is associated with positively enhanced soil physical, chemical, and biological characteristics. In conventional systems, the four important elements of nitrogen, phosphorus, potassium, and calcium are often applied as synthetic fertilizers in relatively heavy concentrations that exceed crop requirements. This can cause soil imbalances in two ways: (1) by increasing or decreasing the availability of some elements essential for crop growth and by changing soil pH, and (2) by increasing productivity over the short-term. On the other hand, deficiencies may occur over the longer term for some other essential elements that are not replaced to meet crop demand. This is in contrast to organic systems, which use organic manures containing minor and trace elements as well as moderate amounts of the primary elements. This balanced fertility in organic farms has beneficial effects leading to fewer problems in disease management (Lampkin, 1999).

Regular inputs of readily soluble fertilizers in conventional agriculture add directly to the pool of immediately available nutrients and, to some extent, bypass soil processes. In organic systems, nutrients are dominantly added to the system as organic (manures, compost, crop residues, legumes) or slow-release sources (e.g., rock phosphate). Most materials incorporated into the soil in organic systems do not contain readily soluble nutrients (K is an exception), and hence a greater reliance is placed on chemical and biological processes to release nutrients in plant-available forms in soil solution (Stockdale et al., 2002).

There are references suggesting that soil on organically managed farms is more fertile with a higher total N, total P, humic acid, exchangeable nutrient cations, water-holding capacity, and microbial biomass than in conventional systems (Wells et al., 2000), and generally contains an average and balanced level of nutrients that have beneficial effects for disease management (Lampkin, 1999).

Nine farms were studied and seven had higher N, six had higher P and three had higher K levels in the organic part compared to conventional part of the farm (Berry et al., 2003). Derrick and Dumaresq (1999) found that soil on an organic farm

contained higher concentrations of exchangeable potassium, calcium, and sodium, and lower concentrations of exchangeable Mn than conventional farms. There were no significant differences between organic and conventional farms in the concentrations of total Mg, Na, N, Mn, and K, nor in exchangeable Mg and organic carbon content.

Joo et al. (2001) found that available phosphorus values were 986 and 935 mg/kg in organic and conventional farm soils, respectively. Average total phosphorus values were 2973 mg/kg in the organic farm fields and 1830 mg/kg in the conventional fields. Oehl et al. (2002) reported that after 21 years of organic management, a balance level of available phosphorus was maintained. An equilibrated input-output budget helps to maintain phosphorus availability at a constant level. Wells et al. (2000) also reported that after three and half years of vegetable cropping, available phosphorous increased on the organically managed field than conventional farms. Fumigation extractable carbon and nitrogen, mineralized N, arginine ammonification, and substrate-induced respiration were significantly higher in organic and low input than conventional systems (Gunapala and Scow, 1998).

However, there are a number of references showing opposite results. Derrick and Dumaresq (1999) observed that the soil of organic farms contained significantly lower concentrations of extractable phosphorus. Loes and Ogaard (1997) and Haraldsen et al. (2000) reported a negative nutrient balance for potassium in the organic cropping systems. The above studies suggest that organically managed farms improve soil nutrient balances and this helps to have fewer disease problems in the long-term.

Various forms of organic amendments and residue management practices contribute to the suppression of plant diseases, although our level of understanding of the mechanisms involved is still limited (Bailey and Lazarivits, 2003). Soil organic matter content and biological activity are generally higher under organic farming systems than conventional systems (Brown et al., 2000; Condron et al., 2000; Daroub et al., 2001; Wells et al., 2000). Joo et al. (2001) found that the average organic matter content was significantly higher, with 44 g/kg in the organically managed farm as opposed to the conventional one, which was 24 g/kg. On the other hand, there are some reports showing no significant differences between organic and conventional systems in terms of total organic matter content (Friedel, 2000).

Published research indicates conclusively that applications of some organic manures increase total phosphorus levels in the soil, and subsequently increase the development of some plant diseases. High total phosphorus level may be due to repeated applications of manure compost with a low N/P ratio (Joo et al., 2001). Shepherd et al. (2002) concluded that for improving soil structure, ephemeral stability through fungal hyphae, extracellular polysaccharides, and (to achieve aggregate stability) frequent input of fresh organic matter is required.

The benefits of applying organic amendments for disease control are incremental and generally slower-acting than chemical fumigants or fungicides, but they may last longer and their effects can be cumulative (Bailey and Lazarovits, 2003). Practices that add organic materials are routinely a feature of organically farmed soils.

Soil microbial communities are strongly influenced by agricultural practices that change the soil environment. Peacock et al. (2001) concluded that soil management practices that result in differential carbon inputs also impact the size and community structure of soil biomass. One such practice is the use of organic amendments and cover crops that increase carbon availability to microorganisms. The dynamics of microbial communities during two growing seasons were compared by Gunapala and Scow (1998) in soils under tomatoes managed by conventional methods with 2- and 4-year rotations, low input, or organic practices. Microbial composition was significantly negatively correlated with amounts of soil mineral N in the conventional system, whereas they were positively correlated with mineral N in the organic system.

Bolton et al. (1985) found that microbial activity and biomass were higher under the organic management system. Another study showed that total bacterial biomass was highest in conventional field soils, while the ratio of active-to-total bacterial biomass was highest in organic field soils (Glenn and Ristaino, 2002). Schjonning et al. (2002) found that after long-term organic management with more than 40 years, microbial biomass C was higher than in conventionally managed dairy farm soils. Carbon released from crop residues contributes to increasing soil microbial activity, and so increases the likelihood of competition effects in the soil.

The placement of the residue in soil can lead to the displacement of the pathogen from its preferred niche, diminishing the pathogen's ability to survive (Bailey and Lazarovits, 2003). Therefore, soil microbial biomass changes as a consequence of switching to organic land management (Shannon et al., 2002), and plant pathogen communities will be different in organic and conventional systems. Moreover, the absence of synthetic pesticides in organically farmed soil has a positive effect on the biodiversity and occurrence of beneficial organisms such as insect pathogenic fungi (Klingen et al. 2002).

Conventional farming systems are often associated with problems such as degradation of soil structure (Jordahl and Karlen, 1993). Brown et al. (2000) reported that conventional farms showed the lowest values for aggregate stability and cation exchange capacity while organic farms had the highest mean humic acid content, air capacity, and available water capacity.

Conventional systems often involve repeated tillage, frequent exposure of bare soil to rainfall, and excessive regular use of fertilizers and pesticides. Such practices can result in severe damage to soil structure, soil erosion, reduced soil fertility, and the loss of fertilizers and other chemicals from increased run-off and leaching (Hollinger et al., 1998) and finally providing a less favorable environment for plant growth and a better environment for plant disease incidences. The conventional farm also suffered from compaction and erosion. In the long-term, these effects can reduce productivity and profitability as the soil resource becomes degraded (Wells et al., 2000). Gerhardt (1997) concluded that an organic farm had a significantly ameliorated soil structure, with an increased A-horizon depth, organic matter content, porosity, earthworm abundance and activity, and better-developed aggregates than the conventional farm.

Organic management methods are able to maintain and improve the structure of the soil in the long-term. Wells et al. (2000) found that after three and half years of vegetable cropping, cation exchange capacity CEC increased in organically managed fields. The soil properties on the organic farms had demonstrated an improved, clear on-site sustainability advantage over conventional systems.

Many secondary plant metabolites function in the defense against plant pests. For example, phenolic metabolites are common constituents of fruits and vegetables that function in the defense against insect and animal herbivory (Stevenson et al., 1993). Conventional agricultural practices utilize levels of pesticides and fertilizers that can result in a disruption of the natural production of phenolic metabolites in the plant (Asami, 2003). There is much evidence to indicate that secondary plant metabolites play critical roles in plant and human health. Plant-based phenolic metabolites are very important due to their potent antioxidant activity and a wide range of pharmacologic properties including anticancer, antioxidant, and aggregation inhibition activity (Rein et al., 2000).

There is a growing concern that the levels of some phenolics may be lower than optimal for human health in foods grown using conventional agricultural practices (Brandt and Mølgaard, 2001, Woese et al., 1997). Differences between the content of secondary plant metabolites in organically and conventionally produced fruits and vegetables allow for the possibility that organically grown produce may benefit plant and human health better than corresponding conventionally grown produce (Asami, 2003).

8 Conclusion

Soil environmental conditions, and especially balanced fertilization, are among the most important components of integrated soil management and crop disease suppression. There is evidence that shows both a positive and a negative relationship between available plant nutrients and the incidence of certain diseases. However, in most cases high levels of nutrients have been shown to causes higher disease incidence. Therefore, it appears that a balanced soil fertility that is normally achieved by application of a proper amount of organic matter could be a practical strategy for prevention of crop diseases.

Soil temperature and moisture are important factors in the development and spread of plant diseases. This is mainly due to the positive effects of the mentioned factors. High temperature, which is normally applied as solarization, has been shown to have detrimental effects on the microorganisms. However, the interactive effect of these factors is also important in disease survival and incidence.

High soil microbial biomass, which is an indicator of soil biodiversity, has been found to affect disease incidence by having a more antagonistic impact on plant pathogens. This could be improved by proper crop management, such as appropriate crop rotation, tillage system, application of mulches, etc.

Soil pH has also been reported to affect the prevalence of disease either positively or negatively. This has been shown to particularly affect soil-borne pathogens

directly or indirectly by the effect on availability of nutrients in the soil. Soil's physical properties, such as temperature, moisture, and structure have been found to affect the abundance and severity of soil-borne diseases through the degree of soil compaction, drainage, and soil temperature.

A variety of organic materials, such as compost and manure, can be used to improve soil structure, food webs, and mineralization of nutrients in the root zone to manage crop diseases in all cropping systems. Almost all crop-production practices influence not only population densities of plant pathogens and disease severity, but also beneficial soil microflora and fauna, as well. The need for replenishing organic matter in the soil after harvesting should be a major concern in present agricultural practices. Such results provide interesting evidence to support the view that long-term management of soil organic matter can lead to better plant resistance against pests and diseases. A concerted research effort is required to address various soil and environmental quality issues associated with the large-scale adoption of farming practices. A comparative study of field soils from organic and conventional farms is needed to better understand the biological, physical, and chemical properties of soil, and their impacts on plant diseases to design a modern integrated pest management (IPM) program. Accumulation of more knowledge regarding the relationships between soil fertility and pests or diseases should stimulate further conversion of conventional crop-production systems to those that incorporate agroecological strategies to optimize soil organic fertilization, crop diversity management, and more natural systems of pest regulation without incurring much yield penalties.

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Soil Protection Through Organic Farming: A Review

Eva Erhart and Wilfried Hartl

Abstract About 17% of the total land area in Europe is affected by erosion, and an estimated 45% of European soils have low organic matter content. Because agriculture occupies the largest proportion of land, agricultural management is decisive for soil conservation and soil quality. Here we evaluate, on the basis of published research, whether or not organic farming might be a way to maintain and restore soil quality. Results of field experiments and studies of practical farms show concordantly that soil organic matter typically increases or is conserved better with organic than with conventional farming practices, with differences becoming exceedingly pronounced with time. Soil organic carbon was 6–34% higher under organic than under conventional management, with two studies finding no pronounced differences and two studies with very old organic farms exhibiting 50–70% more soil organic C than their conventional neighbors. This goes along with an increase in soil total nitrogen content of up to 21% (47% on one of the old organic farms), which nevertheless was shown not to lead to increased nitrogen losses to the groundwater due to nitrogen-conserving practices used in organic farming. In the "plant available" soil contents of phosphorus and potassium, there appears to be no general trend under "organic" as compared to conventional management.

Soil structure is typically positively affected by organic farming practices. There were up to 70% more stable macroaggregates in organic farming, and infiltration rates were up to twice as high as under conventional management. Soil water content increased by 5–72% in the studies analyzed, and an increased soil water content was reported to account for 30% higher yields in the organic systems during the extremely dry years experienced during the Rodale Farming Systems Trial. Erosion, as assessed by measuring topsoil thickness, was lower under organic management, resulting in 2–16 cm thicker topsoils. When the universal soil loss equation

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(USLE) method was used to model erosion, between 15% and 30% less erosion under organic management was reported.

In summary, the research analyzed shows that organic management protects and improves soil quality. The main factors responsible for these benefits were identified as larger inputs of organic matter (manure, compost); more diverse crop rotations, including cover crops and green manures; and a longer time span of soil cover. Because organic farming is the only farming system that is legally defined and controlled, these benefits of organic farming can be relied on, although there is some differentiation within organic farming by different farm types and production intensities.

Keywords Soil organic matter · Nitrogen · Phosphorus · Potassium · Erosion · Aggregate stability · Water infiltration · Water holding capacity

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1 Introduction

Soils fulfill a number of essential functions for man and environment. Soils regulate the natural and human-influenced cycles of water, air, organic matter, and minerals. They filter, buffer, transform, and store materials. Soils are the basic resources and habitat for microorganisms, plants, animals, and humans. And, last but not least, soils are the basis for agriculture and forestry (Schachtschabel et al., 1998). Against this background, it is alarming that soils are under increasing pressure by erosion, decline in organic matter, contamination, sealing, compaction, decline in biodiversity, salinization, floods, and landslides. About 17% of the total land area in Europe is affected by erosion, and an estimated 45% of European soils have low organic matter content (EU Commission, 2006). Soil compaction is perceived as a major risk for soil fertility, particularly in the new EU member states (Hartl 2006). The European Environment Agency estimates economic losses through soil degradation in agricultural areas in Europe at around EUR 53/ha per year, while the costs of off-site effects on the surrounding civil public infrastructure, such as destruction of roads and siltation of dams, reach about EUR 32/ha (EEA, 2003).

Soil degradation is in the public eye only after storms, floods, and wetland slides, otherwise the process of soil degradation proceeds silently and goes unnoticed. Agriculture occupies the largest proportion of land, therefore agricultural management is decisive for soil conservation and soil quality. In organic farming, pesticide pollution—one of the most common problems of conventional agriculture—is completely avoided because pesticides are not used. Moreover, in organic farming, soil quality and health play a crucial role because the fertility of the soil is perceived to not only have implications in terms of soil degradation, but also for the health of the crops, animals, and human beings that derive their sustenance from it, as emphasized by many of the early organic farming protagonists including Eve Balfour, Albert Howard, Newman Turner, Friend Sykes, and Hans Peter Rusch (Lampkin, 2002; Rusch, 1978). The International Federation of Organic Agriculture Movements (IFOAM) lists among the principles of organic farming that "Organic agriculture should be based on living ecological systems and cycles, work with them, emulate them and help sustain them" and "Organic agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible" (IFOAM 2005). The living, healthy soil—as the basis for healthy plants, healthy animals, and healthy products—is considered to be the cornerstone of organic farming (Bio Austria, 2006). Because organic farming is absolutely dependent upon maintaining ecological balance and developing biological processes to their optimum, the preservation of soil structure, earthworms, microorganisms, and larger insects is essential to the working of an organic system. Therefore, the protection of the soil and the environment is a fundamental "must" for the organic farmer, and not something that can be tacked on at the end if profits allow (Lampkin, 2002).

The aim of the present review is to evaluate, on the basis of published research results, if organic farming can live up to its claims to protect the soil and maintain soil quality as compared to conventional farming practices.

2 Soil Organic Matter

The concentration of organic matter in soils is primarily related to climate (temperature and precipitation), to soil texture (clay content), and to soil drainage status. Crop rotation and management usually play a smaller, but important role (Shepherd et al., 2002). In organic farming, great importance is attached to maintaining and increasing soil organic matter because it is perceived as the basis of soil fertility. As, for instance, the principles of the Bio Austria Association put it, "Organic farming strives for a targeted humus management. The input of organic matter must, therefore, at least compensate the losses through decomposition on the long term. Fertilization aims at increasing the activity of soil life." (Bio Austria, 2006).

Several field experiments in the United States and in Europe investigated the changes in soil organic matter and soil organic carbon, respectively, under organic versus conventional management. In the Sustainable Agriculture Farming Systems experiment, an eight-year crop rotation experiment on silty soil in California (United States), organic fertilization with composted and aged manure, cover crops, and a

four-year crop rotation were applied in the organic treatment, while the conventional treatment had a simple two-crop rotation without cover crops, and received mineral fertilizer. After eight years, soil total carbon content, as determined by combustion analysis, in the organic treatment had increased significantly from 9.11 g kg^{-1} to 10.21 g kg^{-1}, while it remained the same in the conventional treatment (Clark et al., 1998). In other experiments, the crop rotation was the same for both organic and conventional treatments.

In the Rodale Institute Farming Systems trial (Pennsylvania, United States), organic management with legumes, and with and without cattle manure, was compared with conventional management with a simple maize–soybean–rotation. After 21 years, the soil content of total carbon, as determined by combustion analysis, amounted to 25 and 24 g kg^{-1} in the organic treatments with and without manure, respectively, and was significantly higher than in the conventional treatment (20 g kg^{-1}) (Fig. 1; Pimentel et al., 2005).

In the Swiss DOK (bio-dynamic, organic, konventionell) experiment, biodynamic management with composted cattle manure and biodynamic preparations and organic management with rotted cattle manure were compared with conventional management, both with and without cattle manure, and with an unfertilized control group. After 21 years, soil organic carbon, as measured by $K_2Cr_2O_7$ digestion, had increased by 1% in the biodynamic treatment, while the soils in the organic treatment and in the conventional treatment with manure had lost 9% and 7% of their C_{org}, respectively. With mineral fertilization, 15% of soil C_{org} was lost, and with no fertilization, 22% was lost (Fliessbach et al., 2007).

Organic management with biowaste compost fertilization was compared to mineral fertilization in an experiment near Vienna, Austria, with a crop rotation of cereals plus potatoes on a Molli-gleyic Fluvisol. After 10 years, soil organic carbon content as determined by combustion analysis had increased from 19.9 g kg^{-1} to 20.5–21.7 g kg^{-1} in the three treatments with increasing rates of biowaste compost

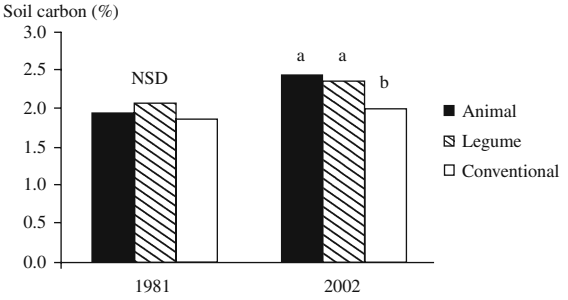

Fig. 1 Percentage of soil carbon for the three systems of the Rodale Institute Farming Systems trial in 1981 and 2002 (organic animal-based cropping, organic legume-based cropping, and conventional cropping). Different letters indicate statistically significant differences according to Duncan`s multiple range test; $p<0.05$. NSD = not significantly different (From Pimentel et al., 2005. Copyright American Institute of Biological Sciences. With permission.)

fertilization, while it remained the same with mineral fertilization and decreased to 18.3 g kg^{-1} without fertilization (Hartl and Erhart, 2005).

Under the more humid conditions of Bavaria, Germany, an experiment was conducted with a cattle manure application plus green manuring, and with mineral fertilization only, respectively. After 15 years, clearly increased carbon contents of 14.3 g kg^{-1} C_{tot} versus 12.8 g kg^{-1} at the beginning were observed with manure plus green manure, while values were unchanged with mineral fertilization (Diez etal., 1991).

The Darmstadt field experiment compared an organic treatment fertilized with rotted manure and liquid manure and a biodynamic treatment that had biodynamic preparations, in addition, with conventional management. The soil organic carbon content, as measured by potassium dichromate digestion, decreased from the starting value of 10.5 g kg^{-1} in all treatments except the biodynamic treatment with the highest application rate. After 18 years, soil C_{org} was 9.5–10.5 g kg^{-1} in the biodynamic treatments, 8.5–10.0 g kg^{-1} in the organic treatments, and 8.0 g kg^{-1} in the conventional treatments (Raupp, 2002).

These findings are supported by comparisons between farms, which have been managed organically and conventionally, respectively, for many decades. Those old organic farms have soil organic matter contents that are 50–70% higher than those of conventional farms. For example, the C_{org} content in the soil of a farm in the Palouse region of Washington, United States, that had been under organic management since 1909, was between 9 and 19 g kg^{-1} (topslope vs. footslope; determined by dichromate oxidation), while the neighboring conventional field had between 6 and 11 g kg^{-1} C_{org} (Mulla et al., 1992; Reganold et al., 1987). In a paired farm study in the Netherlands, with two farms that had been managed organically vs. conventionally for 70 years, a C_{tot} content, as determined by combustion analysis, of 24 g kg^{-1} was measured under organic farming and of 15 g kg^{-1} under conventional farming (Pulleman et al., 2003).

Differences in soil organic matter content, however, do not necessarily take decades to become manifest. They may be observed after several years of organic management. In a survey of 30 farm pairs in southern England, an average C_{org} content, as determined by wet oxidation, of 23.7 g kg^{-1} was measured in the soils of the organic arable farms, and of 17.7 g kg^{-1} on the conventional arable farms (Armstrong Brown et al. 2000). Another study of 14 paired farms in eastern England reported soil C_{org} contents, determined by wet oxidation, of 19.8 versus 15.1 g kg^{-1} on organic and conventional farms, respectively (Munro et al., 2002). In the U.S.-American Corn Belt, Lockeretz et al. (1980) investigated 35 farms that had adopted organic methods roughly nine years before, and their conventional neighbors. Soil C_{org} contents measured by wet oxidation with $K_2Cr_2O_7$ were 23.5 g kg^{-1} versus 22.1 g kg^{-1} on the organic and conventional fields, respectively. In five farm pairs in Nebraska and North Dakota (United States), the average C_{org} content (as determined by dry combustion) was 18.9 g kg^{-1} on the organic farms that had been managed so for more than 12 years, and 15.5 g kg^{-1} in the conventional farms (Liebig and Doran, 1999). The study of Weiss (1990) included 181 organic fields under organic management for on average 12 years, plus adjacent conventional fields in

Baden-Württemberg. On average, the soil C_{org} content determined by potassium dichromate oxidation was 16.9 g kg^{-1} under organic management, and 15.7 g kg^{-1} under conventional management.

There also are, however, a few studies in which no pronounced differences in the humus content between organic and conventional farming were reported. König et al. (1989) investigated two paired farms in the Köln-Aachener Bucht that had been under contrasting management for more than 10 years, both on loess soil of very high natural fertility. There were no general differences in soil organic matter, measured by dry combustion, between organic and conventional farms. The larger plant and root biomass with mineral fertilization (yields were 40% higher) outweighed the effects of manure applications and lucerne-grass used on the organic farms.

In a study comparing 256 paired plots on adjacent organic and conventional fields all over Germany, only a tendency towards higher soil C_{org} contents (measured by dry combustion) with organic management was recorded (Hoyer et al., 2007). Soil microbial biomass, however, was clearly higher with organic management. The lack of differences in soil C_{org} was attributed to the site conditions and the variability in humus reproduction within the organic and conventional farms, respectively.

In summary, comparisons of similar farms under similar soil and climate conditions usually provide a higher soil organic matter content under organic management with differences becoming exceedingly pronounced with time. Besides these effects on total soil organic matter content, many authors have shown that continued addition of organic matter, together with diverse crop rotations, low nutrient input, and (not least) abandoning pesticide use usually increases the size of the microbial biomass and stimulates enzyme activity (Fliessbach and Mäder, 2000; Hansen et al., 2001; Hoyer et al., 2007; Pimentel et al., 2005; Raupp and Oltmanns, 2006; Reganold et al., 1987). Due to the provision of an additional food source, addition of organic matter to soils have also been shown to greatly increase earthworm populations (Haynes and Naidu, 1998; Kromp et al., 1996; Mäder et al., 2002; Maidl et al., 1988; Pulleman et al., 2003; Scullion et al., 2002). Humus composition also changes, as the most stabile humin fraction increases (Fliessbach et al., 2000; Raupp and Oltmanns, 2006; Wander and Traina, 1996).

In summary, results of field experiments and studies on practical farms show concordantly that, while the level of soil organic matter depends to a great extent on site conditions and farm type, soil organic matter typically increases or is conserved better with organic farming than with conventional farming practices, with differences becoming exceedingly pronounced with time. The increase in soil organic matter is due to larger organic inputs such as manure, manure compost, and biowaste compost, and to the more diverse crop rotations that include cover crops on a regular basis.

3 Nitrogen

Increasing soil humus content usually brings about an increase in the total nitrogen content of the soil. In the Rodale Institute Farming Systems Trial (Pennsylvania),

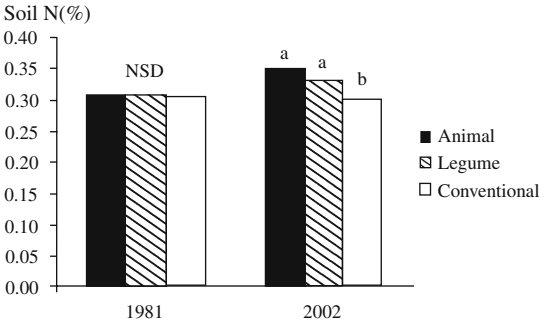

Fig. 2 Percentage of soil nitrogen for the three systems of the Rodale Institute Farming Systems trial in 1981 and 2002 (organic animal-based cropping, organic legume-based cropping, and conventional cropping). Different letters indicate statistically significant differences according to Duncan's multiple range test; $p<0.05$. NSD = not significantly different (From Pimentel et al., 2005. Copyright American Institute of Biological Sciences. With permission.)

organic management with legumes, and with and without cattle manure, was compared with conventional management. The nitrogen content of the soil in the conventional treatment was 3.1 g kg^{-1}, the same as at the start of the experiment, while with organic farming with manure it increased to 3.5 g kg^{-1} and to 3.3 g kg^{-1} without manure after 21 years (Fig. 2; Pimentel et al., 2005). In the DOK experiment in Switzerland (CH), biodynamic management (with composted cattle manure plus biodynamic preparations) and organic management (with rotted cattle manure) were compared with conventional management both with and without cattle manure and with an unfertilized control. Soil total nitrogen content, as determined using the Kjeldahl method, of the biodynamic treatment was 1.63 g kg^{-1}, and significantly higher than that of all other treatments, which ranged from 1.33 to 1.43 g kg^{-1} (Fliessbach et al., 2007).

In an experiment comparing organic management with biowaste compost fertilization to mineral fertilization, the original total nitrogen content, as determined by dry combustion, of the soil of 2.22 g kg^{-1} had decreased to 2.09 g kg^{-1} and 2.12–2.13 g kg^{-1} in the unfertilized and the mineral-fertilized treatments, respectively. With compost fertilization, it decreased to 2.15 g kg^{-1} in the treatment with the lowest compost rate, and increased significantly to 2.30–2.38 g kg^{-1} with higher rates after 10 years (Hartl and Erhart 2005; plus unpublished data). In the paired-farm comparisons of 14 farm pairs in eastern England (Munro et al., 2002), and of five farm pairs in Nebraska and North Dakota (Liebig and Doran, 1999), 21 and 20% higher soil N_{tot} contents were recorded in the organic farms. Munro et al. (2002) compared 14 paired farms in eastern England. Soil N_{tot} contents measured by wet oxidation were 1.08 g kg^{-1} in the conventional and 1.31 g kg^{-1} in the organic farms, on average. In five farm pairs in Nebraska and North Dakota, the average N_{tot} content as determined by dry combustion was 1.52 g kg^{-1} in the organic farms and 1.19 g kg^{-1} in the conventional farms (Liebig and Doran, 1999). Diez et al. (1991), however, did not find statistically significant differences in the total N contents of

the soil between the treatments with cattle manure and green manure and that of mineral fertilization in their experiment.

As a result of increased total nitrogen contents, soil nitrogen mineralization increases. This is desired and necessary in organic farming to feed the crop plants from the soil's resources, but it may also hold the risk of increased nitrogen leaching into the groundwater. Due to the ban on synthetic nitrogen fertilizers, and strict control of purchased organic fertilizers, organic farmers strive to lose as little nitrogen as possible from their soils for ecological, but even more for economic, reasons. Comparisons of the nitrogen balances of organic and conventional farms document the higher nitrogen efficiency of organic farms (Hege and Weigelt, 1991; Van der Werff et al., 1995). Numerous studies compared the nitrogen-leaching potential of organic versus conventional farming practices on the basis of residual N in the soil profile in autumn, nitrogen leaching, and nitrogen concentration in the unsaturated zone (Berg et al., 1999; Eltun, 1995; Erhart et al., 2007; Hartl and Erhart, 2005; Moritz et al., 1994; Pimentel et al., 2005; Stopes et al., 2002). These studies proved smaller nitrogen losses with organic management in most cases; nevertheless, they also showed that some management practices in organic farming—mainly in connection with plowing in legume cover crops—may be problematic in this respect. The time of incorporation of green manure is crucial to avoid leaching losses of N. Organic farmers use several tools to match the nitrogen availability to crop demand in time and space. Cover crops are an important tool in the context of reducing N losses, especially in the second year after incorporating green manure. Differences in the chemical composition of plant species and varieties, i.e., different clover/grass mixtures, may be used to influence the course of decomposition and N mineralization of green manure plant material (Gunnarsson and Marstorp, 2002; Müller et al., 1988; Wivstad, 1999). Deep-rooted, autumn-sown annual crops that have a well-established root system in the spring can access nitrate before it moves below the maximum rooting depth. Deep-rooted crops such as sunflower and safflower, and green manure legumes such as lucerne, may be effective at recovering deep-leached nutrients. Finally, intercropping of legumes and cereals also has the potential to improve nitrogen use (Wivstad et al., 2005).

Results of both field experiments and studies on practical farms typically show an increase in total soil nitrogen content under organic management. This increase does not, however, lead to increased nitrogen losses in the groundwater due to numerous nitrogen-conserving practices used in organic farming, which result in an overall higher nitrogen efficiency of organic farms.

4 Phosphorus and Potassium

Phosphates exist principally as inorganic phosphates in the soil, either as definite phosphate compounds or films of phosphate held on the surface of organic particles. With time, there is a diffusion of the adsorbed phosphorus (P) into the soil matrix, making the P unavailable for uptake in the short-term. Although originally it was thought that P was fixed irreversibly in soils, it is now known that P can move from weak to strong and from strong to weak bonding sites (Stockdale et al. 2002).

Due to the slow rate of diffusion of phosphate, the phosphate supply to a plant depends to a large extent on the size of the root system, the density of its root hairs, the intensity of its ramifications, and the degree of mycorrhization (Lampkin, 2002). Plants, particularly legumes, may mobilize soil phosphates through excretion of protons and acidification of the rhizosphere even in calcareous soils (Trolove et al., 1996). Therefore, cultivating a broad range of crops plus green manures promotes the availability of P (Eichler-Loebermann and Schnug, 2006; Nelson and Janke, 2007; Weiss, 1990).

Lindenthal (2000) investigated phosphorus availability and uptake in long-term ($>$ 40 years) fertilization experiments. He reported a large and lasting mobilization of soil P reserves in the treatments without P fertilization.

In the DOK experiment, where biodynamic management and organic management were compared with conventional management, both with and without cattle manure, and with an unfertilized control group, the flux of phosphorus between the matrix and the soil solution, as determined by the ^{32}P isotopic exchange kinetic method, was found to be highest with biodynamic management (Oberson et al., 1993). Organic P is mineralized by the action of extracellular phosphatases, whose activity has been found to be higher in organic and biodynamically managed soils than in conventional management. In the DOK experiment, soil acid phosphatase activity was 1,714 and 1,635 μg phenol g^{-1} 16 h^{-1}, whereas it was 1,255 in the control and 1,454 and 1,378 μg phenol g^{-1} 16 h^{-1} in the conventional treatments, with and without cattle manure, respectively (Oberson et al., 1993).

Soil microorganisms affect P dynamics by their activity and represent an organic P compartment that can act as a sink and source of plant-available P (Oehl et al., 2001). More phosphorus was bound in the microbial biomass, because the microbial P content released by chloroform fumigation amounted to 17.6 and 16.5 mg kg^{-1} in the biodynamic and organic treatments, respectively, versus 9.0 and 8.0 mg kg^{-1} in the mineral fertilizer and control treatments (Fig. 3; Oehl et al., 2001). Also, the phosphorus flux through the microbial biomass was faster in organic soils. When soil of the DOK experiment was incubated with $^{33}PO_4$ for 56 days, the highest quantity of tracer P was incorporated into microbial P in the biodynamic (17.5%) and organic (11.7%) soils, as compared to 7% in the mineral fertilizer treatment (Oehl et al., 2001).

Any change in nutrient management that increases the returns of organic matter to the system will tend to increase the rates of biologically driven nutrient transformations. SOM also increases the number of low-energy bonding sites available for P, which are critical in regulating short-term P availability (Stockdale et al., 2002).

Soil potassium may be divided into four fractions: soil solution K, exchangeable K, K "fixed" by weathered micaceous minerals ("non-exchangeable K") and K in the lattice of primary minerals. These four fractions constitute a dynamic system with reversible K transfer between the fractions. The potassium supply to plants is not confined to the small amounts of soil solution K and the exchangeable K, but is strongly dependent on soil texture and mineralogy. The root-induced lowering of the K concentration at the root surface may cause a significant release of nonexchangeable interlayer-K (Schachtschabel et al., 1998; Syers, 1998). Up to two-thirds of

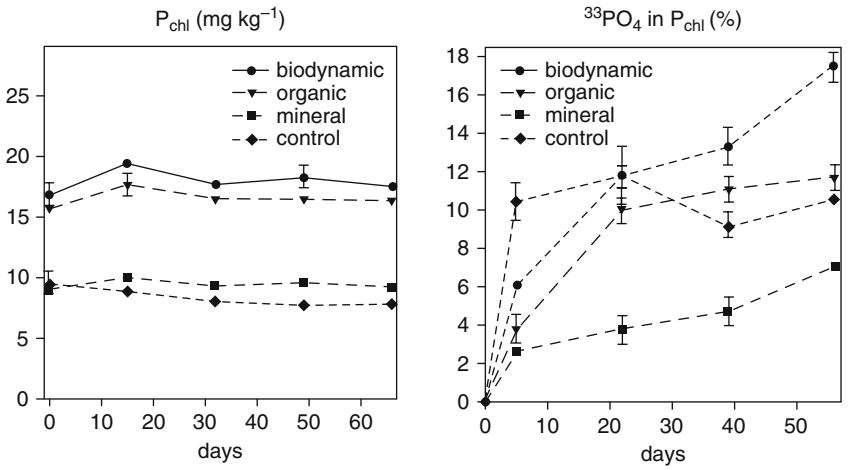

Fig. 3 Microbial $^{31}PO_4$ (*left*) and tracer $^{33}PO_4$ (*right*) released by chloroform treatment (P_{chl}) in the biodynamic, organic, mineral fertilizer, and control treatments of the DOK experiment (From Oehl et al., 2001. With permission from Springer Science + Business Media)

the total K uptake of plants may come from the nonexchangeable pool (Meyer and Jungk, 1992) and according to Wulff et al. (1998), in sandy soils the K content of K feldspar may also be a source of K that is more important to plants than generally assumed. The annual release from the nonexchangeable K pool may amount to 75–100 kg K ha^{-1} (Askegaard and Eriksen, 2002; Wulff et al., 1998).

Potassium uptake by plants is also affected by nutrient antagonisms. High levels of calcium in some soils limit plant K uptake (Brady and Weil, 1999). The K efficiency of crops is related to their rooting intensity. Gramineous plants, which have a high root density, are known to efficiently utilize K from interlayers of clay minerals (Steffens and Mengel, 1979). It has been noticed in some organic systems that very low available K levels, according to soil analyses, are not associated with K deficiency symptoms in the plants or in lower yields, and that additions of mineral K sources under these circumstances do not result in the expected yield increases. This may be due to the fact that the relationship between the different fractions is more finely balanced in an organic system, so any available K is immediately taken up by the growing plant and therefore not reflected in soil analysis figures (Lampkin, 2002). Because soil organic matter contributes a large part to soil cation exchange capacity (CEC), a rise in soil organic matter (SOM), as regularly observed in organic farming, will increase the capacity of the soil to retain cations such as K (Hartl and Erhart, 2003) (Fig. 4).

In the experiments where the organic treatments were fertilized with farmyard manure and compost, respectively, values of plant available P and K were mostly higher than in the treatments with mineral fertilizer (Clark et al., 1998; Diez et al., 1991; Melero et al., 2006), but not always (Mäder et al., 2002). Diez et al. (1991) recorded, that, with time, the availability of P and K had become higher in the treatments with farmyard manure, although the P- and K-balance surpluses were smaller

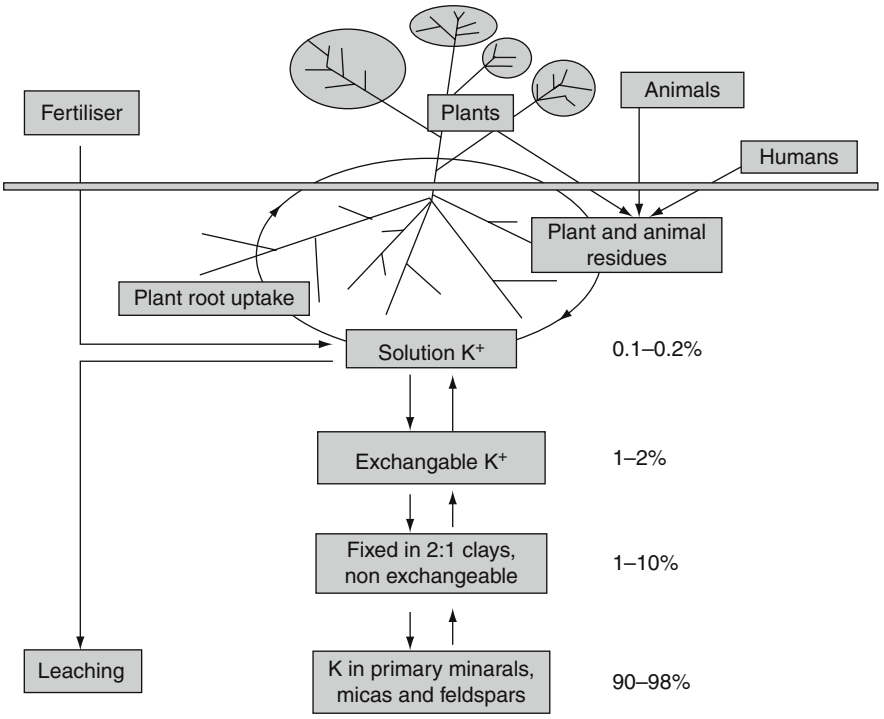

Fig. 4 Major components of the K cycle in the plant-soil system (From Öborn et al., 2005. With permission from Wiley-Blackwell.)

than with mineral fertilizer, which again points to the importance of soil organic matter for P and K availability.

In studies of practical farms, several workers did not find significant differences between organic and conventional holdings concerning available P and K contents of the soil (Liebig and Doran, 1999; Lockeretz et al., 1981; Maidl et al., 1988). Munro et al., (2002) measured 13% higher available P concentrations, but significantly lower available K concentrations in organic fields in England, while König et al., (1989) and Weiss (1990) reported clearly lower values of available P and K on the organic farms investigated in Germany.

There appears to be no general trend in the "plant available" soil contents of phosphorus and potassium in organic farming as compared to conventional farming. Soil analysis figures, however, are of limited value because the P and K supply to plants depends largely on the size of the root system, the rooting density, and the degree of mycorrhization. Increased soil biological activity in organic farming enhances the availability of P. For K, the supply from the "nonexchangeable" pool must not be neglected. To gain a more reliable picture of nutrient supply, soil analyses should be complemented by nutrient balances.

5 Soil Structure

In agronomic terms, a "good" soil structure is one that shows the following attributes: optimal soil strength and aggregate stability, which offer resistance to structural degradation, i.e., capping/crusting, slaking and erosion; optimal bulk density, which aids root development and contributes to other soil physical parameters such as water and air movement within the soil; and optimal water-holding capacity and rate of water infiltration (Shepherd et al., 2002). As shown by Tisdall and Oades (1982), the water stability of aggregates depends on organic materials. The organic binding agents are classified according to their stability into transiently effective compounds (mainly polysaccharides), temporarily effective compounds (roots and fungal hyphae), and persistent compounds such as organomineral complexes and humic acids (Bohne, 1991). The effect of cover crops and green manures is due to the first two groups of substances. With cover crops or green manuring, the living roots bring considerable amounts of dead cells, exsudates, and secretions into the soil, which serve as a food basis for soil microorganisms. The microorganisms breaking down organic matter produce organic binding agents, such as polysaccharides, while the clover and grass roots and fungal hyphae enmesh aggregates and stabilize them in this way (Watson et al., 2002; Beste, 2005). Arbuscular mycorrhizal fungi stabilize soil aggregates through glomalin—a glycoproteinaceaous substance they produce (Nichols and Wright, 2004). Dead roots serve as a substrate for slowly growing microorganisms, and particularly for fungi, even more than litter and crop residues do (Gisi et al., 1997; Beste, 2005). As the effect of these organic binding agents (except for glomalin) is of short to medium duration (weeks to months), frequent addition is required.

In contract, the addition of decomposed or composted materials, such as manure or compost, leads to a more slowly but lasting increase of aggregate stability because, in this case, the organic matter is rich in humic acids, which represent relatively stable binding agents (Haynes and Naidu, 1998). Therefore, a combination of green manures and decomposed or composted materials provides optimum performance (Ekwue, 1992; Raupp, 2002).

Because organic matter plays such a central role in the formation of stable aggregates, there is generally a close correlation between the soil organic matter content and its aggregate stability (Chaney and Swift, 1984; Haynes and Naidu, 1998; Watts and Dexter, 1997). There is a large body of evidence that soil aggregate stability is higher with organic fertilization and in organic farming, respectively.

In the long-term soil organic matter experiment in Ultuna (S), the treatments of bare fallow, no-N, green manure, farmyard manure (well decomposed), and peat have been compared since 1956. The organic fertilizers are applied based on equal amounts of ash-free organic matter. Soil aggregate stability showed a clear response to soil management. It increased in the following order: bare fallow (3.6–6.8% stable aggregates) $<$ no-N (12.3–13.2%) $<$ green manure (29.3–29.4%) $<$ peat (31.1–35.5%) $<$ farmyard manure (36.8–41.5%). Increasing C_{org} contents generally enhanced aggregation, except in the peat treatments, the unfavorably high C/N

ratio and the small size of microbial biomass led to a distinctly lower influence of soil organic matter (Gerzabek et al., 1995).

In the DOK experiment, biodynamic management (with composted cattle manure plus biodynamic preparations) and organic management (with rotted cattle manure) have been compared with conventional management, both with and without cattle manure, and with an unfertilized control group, since 1978. Aggregate stability was determined using two methods. When aggregate stability was measured with a rainfall simulator, the stability of aggregates taken from the biodynamic farming system was highest (33% stable aggregates versus 26–29% in the other treatments). Aggregate stability in percolating water was always higher in both organic farming systems than in the conventional systems. Measured on three dates, it ranged from 533–881 ml 10 min^{-1} in the biodynamic treatment and from 408–937 ml 10 min^{-1} in the organic treatment, compared with 301–745 ml 10 min^{-1} in the conventional treatment with manure and 167–630 ml 10 min^{-1} in the conventional treatment with mineral fertilizer only (Siegrist et al., 1998).

Also in the Rodale Institute Farming Systems trial (Pennsylvania), where organic management with legumes, and with and without cattle manure, was compared with conventional management, enhanced soil aggregation has been observed in both organic treatments following the production of legume hay and cover crops (Werner, 1988; Friedman, 1993; both cited in Wander and Traina, 1996).

In Bavaria, ten pairs of fields under organic management (for at least eight years) versus conventional management were investigated by Maidl et al. (1988). In eight of the pairs, the aggregate stability was higher in the organic fields. On average, the aggregate stability of all pairs was 4% higher in the organic fields. The higher aggregate stability under organic management was attributed to the higher share of grass-clover leys in the crop rotation, and a more shallow soil cultivation on the organic farms. In a paired farm study in the Netherlands, with two farms that had been managed organically vs. conventionally for 70 years, water stable macroaggregation amounted to 75% under organic farming and to 44% under conventional farming (Pulleman et al., 2003).

Two studies comparing paired farms, however, found only little differences in aggregate stability (König et al., 1989; Mulla et al., 1992). This might be due to the stabilizing effects of substances other than organic matter, such as $CaCO_3$, to the threshold value for organic matter in soils, above which there is no further increase in stability, and to the distribution of organic matter in the soil profile (Bohne, 1991; Shepherd et al., 2002).

Soil bulk density may decrease in organic farming as a result of organic matter additions due to a dilution effect caused by mixing of the added organic material with the more dense mineral fraction of the soil (Haynes and Naidu, 1998). In a study of five farm pairs in Nebraska and North Dakota, where the organic farms had been organic for 9–29 years, soil bulk density was an average of 1.26 t m^{-3} in the organic fields as compared to 1.36 t m^{-3} in the conventional fields (Liebig and Doran, 1999). Conversely, increased growing of grass and clover may cause soil bulk density to increase as the soil settles because of the lack of soil cultivation (Maidl et al., 1988). Soil compaction as a consequence of traffic and cultivation of

soils has negative consequences for soil structure and water infiltration, particularly if heavy machinery is used and wet soils are cultivated (Schnug and Haneklaus, 2002).

In organic farming, field passes for (split) fertilizer applications and herbicide/pesticide applications are not required. Instead, there are more field passes necessary for mechanical weed control. The more diverse crop rotations used in organic farming decrease the risk that the soil is cultivated untimely in wet conditions, which may be unavoidable if a large part of the farm is growing the same crop, as in many conventional farms, and cultivation has to be completed in a restricted time frame (Arden-Clarke and Hodges, 1987). Some inorganic fertilizers, such as ammonium sulphate, have a deleterious effect on soil structure by causing dissaggregation of soil clods. A soil that is biologically stabilized may react more dynamically to pressure and become less compressed, or recover more easily, from a short-term compression than a soil that has little biological stabilization (Beste, 2005).

The water regime in soils is influenced by soil organic matter in several ways. First, organic matter increases the soil's capacity to hold plant-available water, defined as the difference between the water content at field capacity and that held at the permanent wilting point. It does so both by direct absorption of water and by enhancing the formation and stabilization of aggregates containing an abundance of pores that hold water under moderate tensions (Ebertseder, 1997; Giusquiani et al., 1995; Haynes and Naidu, 1998; Weil and Magdoff, 2004). Hudson (1994) assessed the effect of the soil organic matter content on the available water content of surface soils of three textural groups. Within each group, as organic matter increased, the volume of water held at field capacity increased at a much greater rate than that held at the permanent wilting point. As a result, highly significant positive correlations were found between organic matter content and available water capacity. As organic matter content increased from 0.5 to 3%, the available water capacity of the soil more than doubled (Hudson, 1994).

Total porosity and pore size distribution for one determine the water-holding capacity of the soil, which may protect crops from water stress in dry seasons, and determine soil water infiltration. In soils with high infiltration rates, water will be absorbed into the soil even in heavy rain, while in soils with low infiltration rates, a portion of the rain will be lost as surface run-off, thus decreasing crop water supply and causing erosion.

Improved soil water-holding capacity is also found in practice with long-time organic management. In the Sustainable Agriculture Farming Systems experiment in California, organic fertilization with composted and aged manure, cover crops, and a four-year crop rotation were applied in the organic treatment, while the conventional treatments had no cover crops and received mineral fertilizer. In the low-input system, fertilizers and pesticides were reduced and cover crops were used. After 10 years, a higher soil water content (27.3%) was measured in the organic system than in the conventional systems (25 and 25.9%, respectively) (Fig. 5; Colla et al., 2000). In the Rodale Farming Systems trial, soil water content was measured during the growing seasons of 1995, 1996, 1998, and 1999 for the organic legume and conventional systems. The measurements showed significantly more

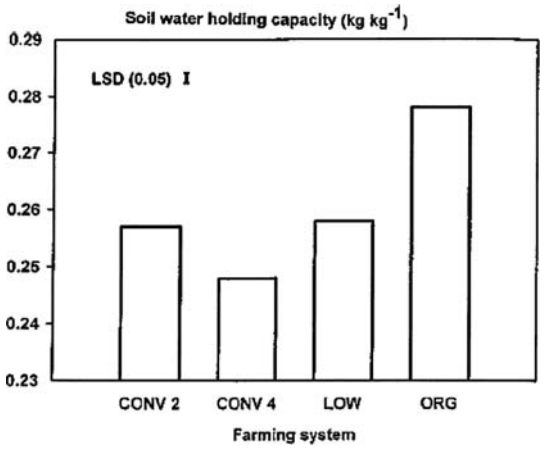

Fig. 5 Mean gravimetric soil water-holding capacity as affected by farming systems in the Sustainable Agriculture Farming Systems project (average of four soil depths and three measurement times). CONV2, conventionally managed two-year rotation; CONV4, conventionally managed four-year rotation; LOW, low-input four-year rotation; ORG, organically managed four-year rotation (From Colla et al., 2000; Copyright by American Society of Agronomy Inc. Reproduced with permission.)

water in the soil farmed using the organic legume system than in the conventional system. This accounted for 28–34% higher yields in the organic treatments in the five extremely dry years during the experiment (Pimentel et al., 2005). In a paired farm study in the Palouse region (Washington), soil moisture contents of 15.5 and 9% were reported from the organic and conventional farms, respectively (Reganold et al., 1987). In five farm pairs in Nebraska and North Dakota, available water-holding capacity was higher in four of the five organic farms. On the average, available water-holding capacity was 0.19 m^3 m^{-3} on the organic farms, and 0.16 m^3 m^{-3} on the conventional farms (Liebig and Doran, 1999). In view of the climate changes envisaged, an improved water supply for the crops may even become more important.

Soil structure, particularly on the soil surface, and soil porosity influence water infiltration capacity. As organic matter improves soil structure and porosity, it also impacts infiltration rate (Shepherd et al., 2002). Earthworms, which are frequently reported to be more abundant in organic farming (Bauchhenss and Herr, 1986; Hofmann, 1995; Kromp et al., 1996; Liebig and Doran, 1999; Mäder et al., 2002), create a great number of macropores (biopores) through their burrowing activity, which is an essential reason for the higher porosity in organically managed soils (Kleyer and Babel, 1984; Schnug and Haneklaus, 2002; Schnug et al., 2004).

Particularly through the favorable conditions for biopores, organically managed fields may have infiltration rates twice as high as in conventional fields. In the Sustainable Agriculture Farming Systems experiment in California, after 10 years soil water infiltration (as measured during 3-h irrigations) was 0.028 m^3 m^{-1} for the conventional, and 0.062 m^3 m^{-1} and 0.065 m^3 m^{-1} for the low-input and organic

systems, respectively, (Colla et al., 2000). In the Rodale Farming Systems trial, infiltration rate was also measured after 10 years and decreased in the order: organic management with legumes and with cattle manure > organic with legumes only > conventional management (Peters et al., 1997).

In summary, soil structure, as measured by soil aggregate stability, water-holding capacity, and water infiltration capacity, is typically positively affected by organic farming through practices such as organic fertilization, cover crops, and green manuring. Higher infiltration rates allow more water to be absorbed by the soil in intense rain, which may mitigate the severity of floods. Increased soil water-holding capacity improves the water supply for the plants during dry seasons, which has been shown to result in significant yield increases during extremely dry years—a feature that may become even more important in view of the climate changes envisaged.

6 Erosion

Soil infiltration capacity is also decisive for the soil`s susceptibility for erosion. Rainwater that cannot infiltrate the soil will run off at the soil surface instead, setting the starting point for erosion (Frielinghaus, 1998). Although it mainly depends on topography and climate, how susceptible a site is to erosion, the kind of land use has a major influence on the start and progress of erosion, the amount of soil eroded, and the degree of damage inflicted.

Numerous cultivation measures in organic farming are capable of reducing erosion—for instance, crop rotations with a high proportion of forage crops and a low proportion of row crops; using cover crops and undersown crops, which keep the soil covered all year round; and the addition of organic matter with all its positive effects on soil structure.

Frequent soil disturbance by mechanical cultivation, which is necessary for weed control in organic farming, however, may increase the risk of erosion, as well as the slower juvenile development of crops, and the premature breakdown of crops due to diseases. Due to lower yields, organic farming also requires larger areas under cultivation than conventional farming (Stolze et al., 2000). Direct seed cropping and no-till farming, which are highly effective in minimizing erosion, are rarely found in organic farming in Europe, because the opinion prevails that they require herbicide use. Results from on-farm experiments in Brazil, however, show that the use of cover crops and crop rotations with legumes may make no-till farming without herbicides technically and economically feasible (Petersen et al., 1999). Reduced tillage systems using the chisel plow instead of the mouldboard plow were tested in several field experiments in Germany, Switzerland, and France. Soil aggregate stability, aeration, and water infiltration capacity were improved with reduced tillage systems (Kainz et al., 2003), although not in all cases (Peigné et al., 2007), and soil humus contents increased (Emmerling and Hampl, 2002; Kainz et al., 2005). The reduced tillage treatments, however, exhibited a tendency towards lower yields (Hampl, 2005; Berner et al., 2008), which became significant in cases where severe weed problems were experienced (Pekrun et al., 2003; Kainz et al., 2005). Practical farmers' experiences show that reduced tillage in organic farming is feasible, but

requires a high degree of competence and committment by the farmer and an adaptation to individual farm conditions (Kloepfer, 2007).

In the paired farm study in the Palouse region, one of the more erosive areas in the United States, the rate of water erosion on the organic farm was 8.3 t ha^{-1} in the organic field and 32.4 t ha^{-1} in the conventional field—almost a four-fold difference. The rate of water erosion on the conventional field was very close to the average annual rate of water erosion in the Palouse area. During the 37 years of organic versus conventional management, the organically farmed silt loam lost about 5 cm of topsoil, while the conventionally farmed silt loam lost about 21 cm of topsoil, leaving a 16 cm difference in topsoil thickness. The differences between organic and conventional management were mainly in the crop rotation (three years including green manuring vs. two years), and in a slightly lower number of cultivations on

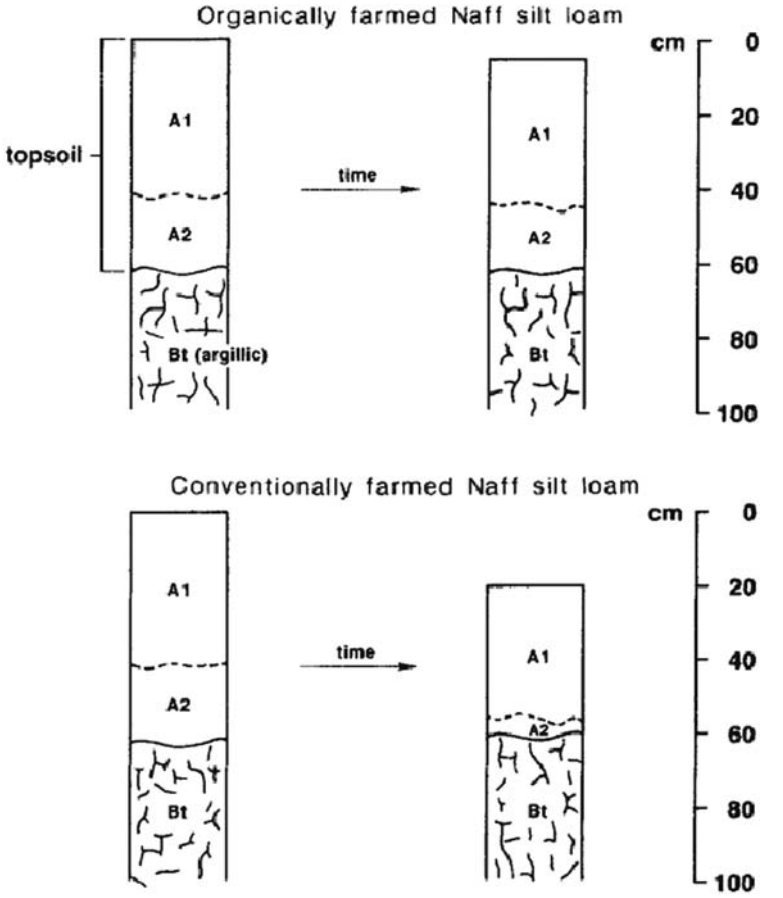

Fig. 6 Organically and conventionally farmed soil losses due to water erosion between 1948 and 1985, in the Palouse region of the United States. A1 is the cultivated topsoil layer. A2 is topsoil below cultivated layer. Bt is subsoil (From Reganold et al., 1987; reprinted by permission from Macmillan Publishers Ltd.: Nature; Copyright 1987)

the organic farm (Fig. 6; Reganold et al., 1987). Topsoil depth was also greater under organic management than under conventional management, albeit only for 2 cm on average, in a study of 14 paired farms in the south and east of England, which represented a wide range of soil texture classes (Munro et al., 2002), and in the three paired farms in Nebraska (here for 2–15 cm) investigated by Liebig and Doran (1999). In a study of 14 paired farms in the U.S. Corn Belt, the effects of differences in crop rotations (but not tillage practices) on erosion were analyzed using USLE. Averaging over all the cropland on each farm, it was estimated that for a given set of physical conditions, water erosion was about one-third less for the rotations used on the organic farms (Lockeretz et al., 1981). In a study in Bavaria, the USLE method was used to model water erosion for 2,056 districts in Bavaria (70547 km^2; 29.8% arable). Detailed cropping statistics and geographical data in a 5 km^2 grid were used. For validation, erosion was measured in 10 subwatersheds on two neighboring farms over eight years. On the average, about 15% less erosion on arable land was predicted for organic farming than for conventional farming due to the larger area of leys, although organic farms more often occupied areas that are susceptible to erosion than conventional farms. The same conclusion was drawn from the validation data (Auerswald et al., 2003).

Since 1993, the research farm Scheyern in Bavaria consists of an organic part and an conventional (integrated) part. In both holdings, numerous measures were taken that succeeded in reducing erosion from 13.2 to 2.0 t ha^{-1} a^{-1} in the conventional part, and from 10.7 to 1.7 t ha^{-1} a^{-1} in the organic part. Still, the annual soil loss is 15% lower under organic management than under conventional (integrated) management (Siebrecht et al., 2007).

These studies of paired organic and conventional farms (both employing their usual tillage practices) and erosion modeling studies show that in organic farming cultivation measures that reduce soil erosion outweigh the factors importing a higher erosion risk, so that in the end, soils under organic cultivation clearly suffer less from erosion.

7 Conclusion

Field experiments and studies on practical farms show concordantly that, while the level of soil organic matter depends to a great extent on site conditions and farm type, soil organic matter typically increases (or is conserved better) with organic than with conventional farming practices, with differences becoming exceedingly pronounced over time. In the studies analyzed, the plus of soil organic carbon under organic management ranged from 6 to 34%, with two studies finding no pronounced differences and two studies with very old organic farms exhibiting 50–70% more C_{org} than their conventional neighbors. The increase in soil organic matter is due to larger organic inputs such as manure, manure compost, and biowaste compost, and to the more diverse crop rotations that include cover crops and green manuring on a regular basis.

The increase in soil organic matter under organic management brings about an increase in soil total nitrogen content of up to 21% (47% in one of the old organic farms) in the studies analyzed. This increase was shown not to lead to increased nitrogen losses to the groundwater due to a variety of nitrogen-conserving practices used in organic farming, which result in an overall higher nitrogen efficiency of organic farms.

In the "plant available" soil contents of phosphorus and potassium, there appears to be no general trend in organic farming as compared to conventional farming. Soil analysis figures, however, are of limited value as the P and K supply to plants depend largely on the size of the root system, the rooting density, and the degree of mycorrhization. Increased soil biological activity in organic farming was shown to enhance the availability of P. For K, the supply from the "nonexchangeable" pool must not be neglected.

Soil structure, as measured by soil aggregate stability, water-holding capacity, and water infiltration capacity, is typically positively affected by organic farming practices. The water-stability of aggregates depends on organic materials such as polysaccharides and humic acids, and the physical enmeshing of aggregates by roots and hyphae, all of which is provided by organic fertilization, cover crops, and green manuring. In the studies analyzed, the effects of organic farming ranged from no effect to more than 70% more stable macroaggregates.

Soil water infiltration is influenced by soil structure, particularly on the soil surface, and by soil porosity. Particularly through the favorable conditions for earthworms, and the macropores they create, organically managed fields may have infiltration rates twice as high as in conventional fields, as measured in the Sustainable Agriculture Farming Systems experiment. Higher infiltration rates allow more water to be absorbed by the soil in intense rain, which may mitigate the severity of floods.

The soil's capacity to hold plant available water is increased by organic matter through direct absorption of water and by the stabilization of aggregates that contain an abundance of pores. Soil water content was increased by 5–72% in the studies analyzed, and an increased soil water content was reported to account for 30% higher yields in the organic systems in the extremely dry years experienced during the Rodale Farming Systems trial. Available water-holding capacity, as measured in one study, was 19% higher in the organic farms there. Improving the water supply for plants in dry seasons is a feature that may become even more important in view of envisaged climate changes.

Erosion was assessed in several studies by measuring topsoil thickness. The organically farmed soils had clearly lost less topsoil through erosion, resulting in 2–16 cm more topsoil left. In other studies the USLE method was used to model erosion, and between 15 and 30% less erosion under organic management was reported. Both methods showed that in organic farming, cultivation measures that reduce soil erosion outweigh the factors importing a higher erosion risk, so that in the end, soils under organic cultivation clearly suffer less from erosion.

In summary, the research analyzed shows that organic management protects and improves soil quality. The benefits of organic farming shown in this article can normally be relied on, although there is some differentiation within organic farming by

different farm types and production intensities. Nevertheless, organic farming is the only farming system that is legally defined, and in which the farmers' adherance to the regulations is strictly controlled. Therefore, the benefits that are inherent to organic farming are, to a certain degree, guaranteed to arise.

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Surfactants in Sludge-Amended Agricultural Soils: A Review

Alicia Fernández Cirelli, Carlos Ojeda, Mariano J.L. Castro and Miquel Salgot

Abstract Surfactants are included in different detergent formulations and are one of the most ubiquitous and important families of organic compounds. The possible contamination of the environment by surfactants is due to their remarkable capacity to modify the surface tension of water and solubilize xenobiotic subtances. Detergents are a common component of domestic wastewater, which may be disposed in water bodies without treatment, with possible health risks through drinking water. Although the generic term "surfactants" is applied to a great number of products, 80% of their demand is covered by only 10 types of compounds. The global surfactant market volume size is more than 18 million tons per year. Large quantities of surfactants are continuously released to the environment, accumulating in sediments and soils, where they can or cannot be degraded depending on their structure. Linear alkylbenzenesulfonate (LAS) is the most widely used surfactant. LAS can be degraded under aerobic conditions, but is persistent in the environment under anaerobic conditions. Surfactants may enter the terrestrial environment through several routes, by far the most important being the use of sewage sludge as fertilizer on agricultural land. High concentrations of surfactants and their degradation products may affect the biota. On the other hand, due to their amphiphilic nature, surfactants may interact both with inorganic as well as organic contaminants affecting their bioavailability. Sections of this review article include: uses, types, and consumption of surfactants, analysis of surfactants in environmental matrices, surfactants in sludge-amended soils, biodegradation of surfactants, transport and fate of surfactants in wastewater treatment plants, fate of surfactants in waters and soils, and interaction of surfactants with soil contaminants.

Keywords Surfactants · Environment · Degradation · Agricultural · Bioavailability · Sewage sludge · Soap · Soil

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1 Introduction

Surfactants, abbreviated from "surface-active agents" are major component of soap shampoo, together with subsidiary components such as builders, e.g., tripolyphosphate, boosters, and auxiliary compounds, included in the formulation of detergents (Smulders, 2001; Kreinfeld and Stoll, 1977). The main use of this kind of formulation is to remove fatty subtances from difference surfaces such as the human body, hair, clothes, or dishes. In terms of environmental issues, the focus of concern is largely on the effects of surfactants from detergent formulations in ecosystems, although there was a period several years ago when the increasing use of builders also presented environmental problems, until the introduction of restrictive legislation.

The presence of both hydrophobic and hydrophilic groups in each molecule is a fundamental physical property of surfactants that allows these compounds to form micelles in solution. It is the formation of micelles in solution that gives surfactants their detergency and solubilization properties. The concentration of surfactants in water in which surfactant molecules aggregate into clusters (micelles) is known as the critical micelle concentration (CMC) (Rosen, 2004).

Historically, the potential pollution of the environment due to surfactants followed the shift from the use of soap-based detergents to synthetic surfactants (Hill et al., 1997). The transition period was approximately from 1940 to 1970, when the use of synthetics rose about three orders of magnitude, while the use of soap fell to less than a half. During this time, there was also a partial transition from the use of solid domestic detergents (powders) to liquids. Until 1960, the major surfactant used in detergency was propylene tetramer benzene sulphonate. It was about this time that sewage treatment problems began to arise and foaming problems appeared in wastewater treatment plants and on rivers. Propylene tetramer benzene sulphonate was being discharged into water systems and was found to be resistant to

biodegradation by bacteria due to the branched alkyl chain. The prohibition of use of this nonbiodegradable surfactant forced the switch to more biodegradable straight-chain alkyl surfactants, and now the major anionic surfactant used today is linear alkylbenzene sulphonate (LAS) (Fischer, 1982; Balson and Felix, 1995; Swisher, 1987).

After use, large quantities of detergents and their components are released into aquatic and terrestrial environments. LAS may enter the terrestrial environment by several routes, mainly through sludge amendments and pesticide applications. It should be noted that formulation of pesticides for crop protection also include surfactants, as happens with detergent formulations. However, in the last few decades, the use of sewage sludge as fertilizer on agricultural land has been by far the dominating input for soils. The load of LAS in sewage sludge may be considerable, with concentrations of more than 10 g/kg dry weight (Jensen, 1999). Therefore, LAS and its metabolites may appear in appreciable concentrations in sludge-amended soils. On the other hand, it has been estimated that 5% of LAS produced in the United States reach the aquatic environment (Liwarska-Bizukojc et al., 2005), where concentrations at μg/L levels have been found, mainly due to the discharge of wastewater treatment effluents into surface waters.

The increasing use of sludge as a soil organic amendment and pesticide application points out the relevance of the study of the behavior of surface-active substances in agricultural soils. A review of the literature concerning the characteristics of surfactants, their transport and transformations in wastewater treatment plants, and their fate once in the terrestrial environment is provided.

2 Uses and Consumption of Surfactants

Surfactants are one of the most widely used families of organic compounds, being used in different formulations in a lot of industries like cosmetics, personal care, household products, paint, coating, textiles, dyes, polymers, food, agrochemical supplies, and oils. A fundamental property of surfactants is their ability to form micelles in solution. This property is due to the presence of both hydrophobic and hydrophilic groups in each surfactant molecule. At concentrations above the critical micelle concentration (CMC) level, surfactants have the ability to solubilize more hydrophobic organic compounds than would be dissolved in water alone.

2.1 Types of Surfactants

Surface-active agents have a characteristic molecular structure consisting of a structural group that has a very little attraction to water, known as a hydrophobic group, together with a group that has strong attraction for water, called hydrophilic group. This is known as an amphiphilic structure. The hydrophobic group is usually a long-chain hydrocarbon, and the hydrophilic group is an ionic or highly polar group. According to the nature of the hydrophilic group, surfactants are classified

Table 1 Acronyms of surfactants

Acronym	Name
LAS	Linearalkylbencenesulfonate
LES	Lauryl ether sulfate
FAS	Fatty alcohol sulphates
FAES	Fatty alcohol ethoxylate sulphates
CTAC	Cetyl trimethyl ammonium chloride
DODAC	Dioctadecyl dimethyl ammonium chloride
AEO	Alcohol ethoxylates
APEO	Alkylphenol ethoxylates
FAEO	Fatty acid ethoxylates
APG	Alkylpolyglycosides
CAPB	Cocamidopropyl betaine
CAHS	Cocamidopropyl hydroxysultaine

as: anionic, cationic, nonionic, and amphoteric. Most-used surfactants and their acronyms are shown in Table 1.

The hydrophilic and hydrophobic groups of the different types of surfactants, as well as the structures of the most important compounds, are shown in Table 2.

2.2 Uses of Surfactants

Surfactants are one of the most ubiquitous and important families of organic compounds. In fact, life on earth is possible because special kinds of surfactants are present in all living-cell membranes. Surfactants in different formulations are being used in many industries like cosmetics, personal care, household, paints, coatings, textiles, dyes, polymers, foodstuff, agrochemical supplies, oils, and in relation to environmental care in applications like wastewater treatment (Castro et al., 2005). A brief summary of surfactant development starting with soap, whose manufacture was described by the Sumerians as long ago as 2500 BC (Smulders, 2001), is shown in Table 3.

Surfactants are widely used because of their two essential properties: their ability to reduce the surface or interfacial tension, and their capacity to solubilize water-insoluble compounds. At present, the generic term "surfactant" applies to a great number of surface-active products. However, 80% of their demand is covered by a group of less than 10 types of products, and the main ones are LAS, lauryl ether sulfate, and alcohol ethoxylates; the surfactant that still has the highest consumption worldwide is soap.

The global surfactant market volume size is about 18 million tons (2003), with an overall rough value of 13 billion Euros (approx. U.S.$19 billion). North America is the biggest surfactant market in the world with 35% of the total; Asia-Pacific follows next with 29%; Western Europe consumes 23%; and the rest of the world accounts

Table 2 Types of surfactants

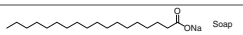
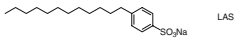
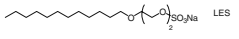
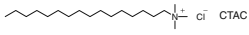
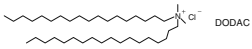
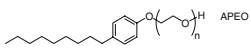
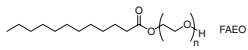
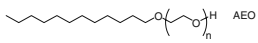
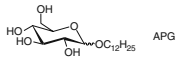
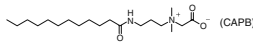
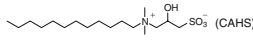
Surfactants	Alkyl Tail	Polar Head	Example
Anionic	C_8—C_{20} straight or branched-chain	—COOH	 Soap
	C_8—C_{15} alkylbenzene residues	—SO_3Na	 LAS
	C_8—C_{20} straight-chain ethoxylated	—OSO_3Na	 LES
Cationic	C_8—C_{18} straight-chain	—$N(CH_3)_3Cl$	 CTAC
	C_8—C_{18} straight-chain	—$N(CH_3)_2Cl$	 DODAC
Non-ionic	C_8—C_9 alkylphenol residues	—$(CH_2CH_2O)_n$—OH n: 4–22	 APEO
	C_8—C_{20} straight of branched-chain	—$COO(CH_2CH_2O)_n$—OH n: 4–22	 FAEO
	C_8—C_{20} straight of branched-chain	—$(CH_2CH_2O)_n$—OH n: 2–22	 AEO
	C_8—C_{20} straight of branched-chain	glucose	 APG
Amphoteric	C_{10}—C_{16} amidopropylamine residue	—$N^+(CH_2)_2CH_2COO^-$	 (CAPB)
	C_8—C_{18} straight-chain	—$N^+(CH_2)_2CH_2CH(OH)CH_2SO_3^-$	 (CAHS)

Table 3 Surfactant landmarks

Year	Surfactants development
2500 BC	Sumerians manufactured soap
1907	Soap commercial manufacture
1928	First anionic synthetic detergent
1946	Branched alkylbenzene sulphonate
1948	Non-ionic surfactants
1950	Cationic surfactants
1954	Anionic-nonionic combinations
1960	Linear alkylbenzene sulphonate (LAS)
1991	Gemini surfactants (Zana and Xia, 2004)

for 13%. The global surfactant market considered by end-user applications shows that 40% of the market is household detergents, followed by textile auxiliaries, and personal care products (Hauthal, 2004).

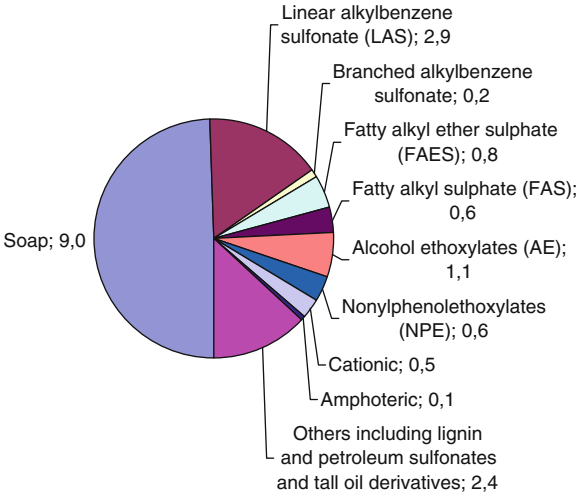

Linear alkylbenzene sulfonate (LAS); 2,9
Branched alkylbenzene sulfonate; 0,2
Fatty alkyl ether sulphate (FAES); 0,8
Fatty alkyl sulphate (FAS); 0,6
Alcohol ethoxylates (AE); 1,1
Nonylphenolethoxylates (NPE); 0,6
Cationic; 0,5
Amphoteric; 0,1
Others including lignin and petroleum sulfonates and tall oil derivatives; 2,4
Soap; 9,0

Fig. 1 World surfactants consumption in millons of tons (2003). Adapted from Liwarska-Bizukojc (2005)

The surfactant volume forecast for the household detergent sector estimated for the years 2001–2012 showed an increase of 20% consumption for the industrialized countries in comparison with 70% for future demands in Asia-Pacific countries (Liwarska-Bizukojc et al., 2005). The world surfactants consumption in 2003 is shown in Fig. 1.

Anionics are the earliest and the most common surfactants not only used as detergents, but also widely applied in many fields of technology and research. They are usually considered to be the "workhorse" in the detergency world. They have been successfully employed to enhance the efficacy of the active ingredients in pharmaceutical and agriculture formulations, cosmetics, biotechnological compounds, and several industrial processes. In some countries, particularly in the Asia-Pacific region and Latin America, the ecologically questionable branched alkylbenzene sulfonates are still in use. However, due to their limited biodegradability, it is only a matter of time before they are substituted by the already dominant linear type (LAS).

The increased use of dishwashing liquids, shampoos, and surfactant-based bath preparations (mainly in Asia) are the major contributory factors to the annual growth rate of 4.5% for lauryl ether sulfate. Fatty alcohol sulphates will undoubtly also increase in importance due to the substitution of traditional soap by synthetic surfactants offering higher performances.

Nonionic surfactants have low sensitivity to water hardness and pH and are frequently used in mixtures with ionic surfactants, resulting in beneficial associations. One of the major reasons for an annual growth rate of 4% for alcohol ethoxylates is the substitution of ecologically questionable alkylphenol ethoxylates, which are still being used in some parts of the world.

Carbohydrate-based surfactant production is expected to grow visibly in the next few years. Alkylglycosides (APG, Table 2) are nonionic surfactants with

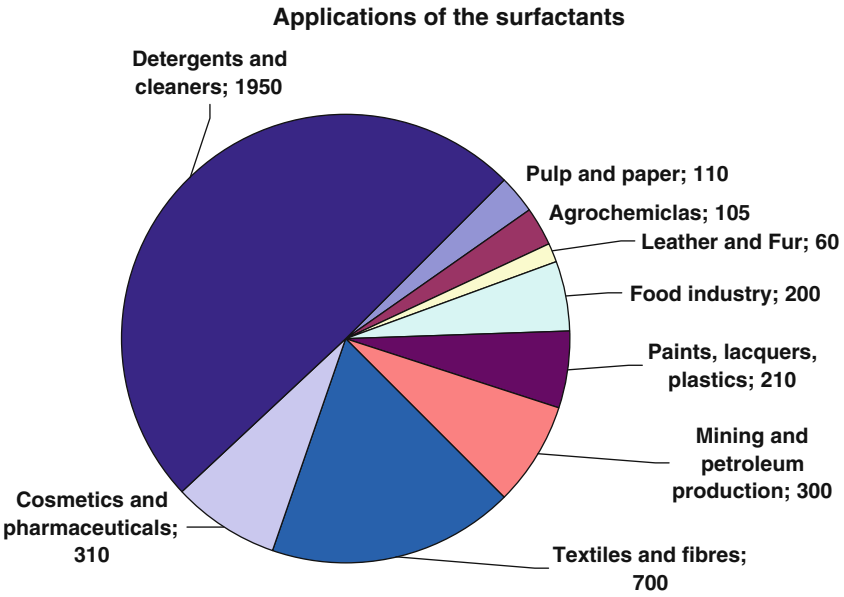


Fig. 2 Surfactant consumption (millons tones) in the USA, Japan and Western Europe. Adapted from Scott and Jones (2000)

remarkable properties (Menger and Keiper, 2000). In industrial scale, they are prepared from fatty alcohols and carbohydrates and are gradually replacing the other known nonionic surfactants derived from the petrochemical industry. Combinations of fatty alcohol sulphates with alkylglycosides in dishwashing liquid formulations are expected to prevail in the market. There is an increasing demand for alkylglycosides, both in the detergent market and in the cosmetics field. Due to their excellent biodegradability and the absence of toxic effects, food elaboration, polymer manufacture, and the solubilization of biological membranes are some of the wide spectrum of their possible applications (Kreinfeld and Stoll, 1997).

In accordance with the different types of industries, Fig. 2 outlines the major uses of surfactants in the United States, Japan, and Western Europe (Scott and Jones, 2000).

2.3 Analysis of Surfactants in Environmental Matrices

The analytical methods for the determination of surfactants in environmental matrices have been continuously improved in regards to reproducibility, selectivity, and sensitivity over the last few years. The main problem in the analysis of surfactants is that they tend to concentrate at interfaces due to their amphiphilic nature. Consequently, losses of surfactants from aqueous solutions occur because of their adsorption onto laboratory apparatus or suspended particles. Especially for matrices like

sewage sludge, sediments, and biological samples, the quantitative recovery of the analytes becomes a major problem. Fertilization of agricultural land with sewage sludge (see Section 3) has resulted in the need to monitor surfactant concentrations in sludge-amended soils. Samples are usually collected from the soil's upper 5 cm with a stainless steel corer, dried at 60°C, pulverized, and stored at 4–8°C in the dark (Marcomini et al., 1989a).

The method of choice for the extraction of surfactants from sewage sludge, sediments, and soils is solid-liquid extraction. In most cases, however, further purification of the extracts is necessary prior to quantitative determination. LAS are desorbed from sewage sludge, either in a noncontinuous procedure by extraction into chloroform as ion pairs with methylene blue (McEvoy and Giger, 1986), or in a continuous procedure by the application of a Soxhlet apparatus and addition of solid NaOH to the dried sludge to increase extraction efficiency (Marcomini and Giger, 1987). Heating of sludge or sediment samples in methanol under reflux for 2 h is also sufficient to extract LAS with recoveries of 85% (Matthijs and De Henau, 1987).

The concentrations of surfactants in environmental samples are usually below the limit of the analytical method. Therefore, preconcentration is necessary before analysis. Interfering substances from the matrix have to be removed in an additional prepurification step prior to quantitative determination of the surfactants.

Anionic surfactants are efficiently concentrated at reversed-phase (RP) materials consisting of silica gel modified with alkyl groups of different chain lengths or graphitized carbon black (GCB). LAS have been extracted by C2- (Field et al., 1992), C8- (Marcomini and Giger, 1987; Trehy et al., 1990), or C18-silica gels (Kikuchi et al., 1986; Leon et al., 2000; Heinig et al., 1998; Sarrazin et al., 1997).

Marcomini et al. (1993a) developed a method for the simultaneous determination in water of LAS and nonylphenol ethoxylates (APEO) as well as their metabolites sulfophenyl carboxylates (SPC) and nonylphenoxy carboxylates (NPEC), respectively. Wastewater or river water samples are adjusted to pH 2 with HCl and passed through C18 cartridges. The adsorbed analytes are eluted with methanol.

The earliest attempts to analyze surfactants in the environment relied on nonspecific analytical methods, such as colorimetry and titrimetry. The main disadvantage of these methods is that, apart from surfactants, other interfering organic compounds from the environmental matrices are recorded too, resulting in systematic errors. Nevertheless, colorimetric and titrimetric methods are still widely used for the determination of anionic, nonionic, and cationic surfactants because of their easy handling and the need for relatively simple equipment.

Anionic surfactants are determined with methylene blue. The procedure is based on the formation of ion pairs between the cationic dye methylene blue and anionic surfactants, which are extractable into chloroform. The concentrations of anionic surfactants are determined colorimetrically at 650 nm after separation of the organic phase (DIN, 1980). Other anionic organic compounds also form extractable complexes with methylene blue, resulting in high values for methylene blue active substances (MBAS). On the other hand, a problem with the previous method is the

presence of cationic substances, which lead to low values because of formation of ion pairs with anionic surfactants.

The ultimate goal in detergents' environmental analysis is the quantification of individual compounds separated from all their isomers and/or homologues. Chromatographic methods like high-performance liquid chromatography (HPLC), gas chromatography (GC), or supercritical fluid chromatography (SFC) are among the most powerful analytical instruments with regard to separation efficiency and sensitivity. Because of the low volatility of surfactants, HPLC is used far more often than GC. Since the launch of atmospheric pressure ionization (API) interfaces, liquid chromatography-mass spectroscopy (LC–MS) coupling is increasingly used for determination of surfactants.

The majority of HPLC applications in the determination of anionic surfactants are only concerned with the analysis of LAS—the most used surfactants in present detergent formulations. Individual homologues of LAS are typically separated on reversed-phase columns with a $NaClO_4$-modified mobile phase using ultraviolet or fluorescence detection. Application of C18 columns with gradient elution results in the separation not only of the LAS homologues but also of their isomers (Matthijs and De Henau, 1987; Marcomini and Giger, 1987; Marcomini et al., 1989b; Vogt et al., 1995). However, short-chain alkyl-bonded reversed phases like C8 (Marcomini and Giger, 1987; Ahel and Giger, 1985a; Leon et al., 2000; Ceglarek et al., 1999; Di Corcia et al., 1991) and C1 columns (Castles et al., 1989), or long-chain C18 phases with isocratic elution (Nakae et al., 1980; Holt et al., 1989) eluted the isomers of every single LAS homologue as one peak. Thus, the interpretation of the chromatograms becomes easier because of a greatly reduced number of peaks. Fluorescence detection is more selective and more sensitive than ultraviolet detection, resulting in lower detection limits. Detection limits of 2 μg/L for water using fluorescence detection (Castles et al., 1989), compared to 10 μg/L for water using ultraviolet detection, have been reported for determination of LAS by HPLC.

Simultaneous determination of LAS and their main metabolites (sulfophenyl carboxylates) was enabled by LC–MS with an electrospray ionization (ESI) interface. Problems with high salt loads of the mobile phase due to the ion pair reagent have been overcome by the incorporation of a suppressor between the LC column and the mass spectrometer (Knepper and Kruse, 2000).

An LC–MS method for the determination of lauryl ether sulfate and fatty alcohol sulphates was introduced by Popenoe et al. (1994). After separation on a C8 column, the analytes are determined by ion spray LC–MS. The mass chromatograms obtained give information about both the distribution of the alkyl homologues and the distribution of the oligomeric ethoxylates, as well.

The main nonionic surfactants, as indicated before, are alcohol ethoxylates, alkylphenol ethoxylates, and (recently) alkylpolyglycosides. The hydrophobic part of alcohol ethoxylates consists of n-alkanols with chain lengths between 8 and 20; typical alkylphenol ethoxylates are branched-chain octyl- or nonylphenol; and alkylpolyglycosides typically have alkyl groups with chain lengths in the range of 8–18. The degrees of polymerization of the polyethoxylate chains of alcohol

ethoxylates and alkylphenol ethoxylates vary from 3 to 40 ethoxy units, while the average polymerization degree of alkylpolyglycosides is in the range of 1.3–1.7 moles glucose per mole of fatty alcohol. Giger et al. (1984) described a reversed-phase HPLC method for the determination of alkylphenol ethoxylates on a C8 column with isocratic water/methanol elution and ultraviolet detection at 277 nm. Under these conditions, the homologous compounds of the alkylphenol ethoxylates series are separated into two peaks. Normal phase HPLC is mostly applied to obtain information about the ethoxylate chain distribution of alkylphenol ethoxylates. Aminosilica columns with gradient elution and ultraviolet detection are well-suited to determine the individual oligomers of alkylphenol ethoxylates (Ahel and Giger, 1985a, b; Marcomini and Giger, 1987).

Fluorescence detection is also used for the simultaneous determination of LAS and alkylphenol ethoxylates, as well as their corresponding metabolites—sulfophenyl carboxylates and nonylphenoxy carboxylates, respectively—by reversed-phase HPLC and gradient elution (Marcomini et al., 1993a, b).

HPLC analysis of alkylpolyglycosides has also been carried out with C8 (Steber et al., 1995) or C18 columns by use of a refractive index detector (Spilker et al., 1996), or a conductivity detector after the addition of 0.3 mol/L NaOH to the eluate in a postcolumn reactor (Steber et al., 1995).

Several LC–MS methods using an electrospray ionisation (ESI) interface have been published for the analysis of alkylphenol ethoxylates and alcohol ethoxylates. The formation of crown ether-type complexes between the ethoxylate chain and cations like NH_4^+ or Na^+ leads to efficient ion formation of the alkylphenol ethoxylates and alcohol ethoxylates surfactants during the electrospray process (Loyo-Rosales et al., 2003; Ferguson et al., 2001; Cohen et al., 2001). By using a C18 HPLC column, nonylphenoxy carboxylates and alcohol ethoxylates are separated according to their aliphatic chain lengths. In the subsequent mass analysis, coeluting ethoxylate homologues are individually detected because of their differences of 44 mass units (CH_2CH_2O, m/z 44) (Cohen et al., 2001).

3 Surfactants in Sludge-Amended Soils

Surfactants find applications in almost every chemical industry, such as personal care, household, agrochemicals, paints, mining, petroleum, paper. Laundry detergents, cleaning agents, and personal care products are by far the largest class of surfactant-containing products for domestic use. After use, they are mainly discharged into municipal wastewater that enters sewage treatment plants. On the other hand, agricultural pesticides have to be formulated to include surfactants to dissolve the active compounds into a hydrophilic system and, in part, they are discharged directly into the soil or reach it after some time because of rain or irrigation water. The different ingredients of a detergent formulation are stopped, modified, or eliminated there by biodegradation or adsorption. Consequently, in the case of

insufficient biological degradability, they are potential sources of environmental pollution.

3.1 Biodegradation of Surfactants

Surfactants can be degraded mainly under aerobic conditions. Some of them are persistent under anaerobic conditions, such as LAS. Alkylphenol ethoxylates are partially degraded in anaerobic conditions to form nonyl and octyl phenols, which are persistent and have shown estrogenic activity to organisms such as fish. High concentrations of surfactants and their degradation by-products may affect the biota. The environmental risk posed by surfactants and their degradation by-products can be assessed in terms of toxicity based on the comparison of the predicted environmental concentration and the predicted no-effect concentration. Nevertheless, more toxicity data are needed for terrestrial risk assessment of surfactants and their degradation products (Guang-Guo, 2006).

LAS are generally regarded as biodegradable surfactants. It should be noted that the very high levels of biodegradation (97–99%) have been found in some wastewater treatment plants (WWTP) that use aerobic processes. In contrast, alkylphenol ethoxylates are less biodegradable (0–20%) (Swisher, 1987). The breakdown mechanism of LAS involves the degradation of the linear alkyl chain, the sulphonate group, and finally the benzene ring. The biodegradation pathways for LAS have been reviewed (Swisher, 1987; Schöberl et al., 1988) and it is shown, as stated previously, that in general terms, their degradation pathway may be split into four different processes:

1) Oxidative conversion of one or two methyl groups of the alkyl chain into a carboxyl group (ω-oxidation).
2) Oxidative shortening of the alkyl chain by two carbon units β-oxidation.
3) Oxidative ring splitting.
4) Cleavage of the carbon-sulphur bond—i.e. sulphate liberation.

Many bacteria and a few fungi are reported to be able to partly degrade LAS. The complete biodegradation of surfactants requires a mixture of bacteria due to the limited metabolic capacities of individual microorganisms. The biodegradation of LAS requires a four-member consortium, three members of which oxidize the alkyl chain, but synergism among the four members is essential for mineralization of the aromatic ring. The breakdown of the alkyl chain starts with the oxidation of the terminal methyl group to form sulfophenyl carboxylates (SPC). Degradation rates are faster for the longest alkyl chain LAS, and slower for LAS isomers with the sulfophenyl group situated in the middle of the alkyl chain (Fig. 3).

Perales et al. (2003) corroborate the metabolic route of LAS biodegradation proposed by several authors, in which the LAS first undergoes oxidation of the extreme terminal of the alkyl chain with the consequent formation of sulfophenyl

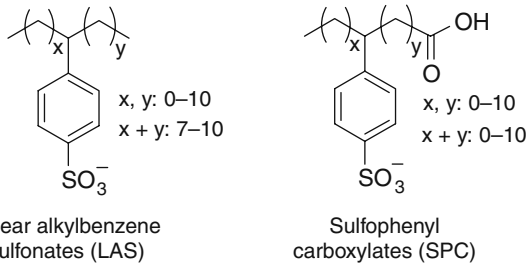


Fig. 3 General structure of linear alkylbenzene sulfonate and sulfophenyl carboxylate

carboxylates of long chain, and subsequently a successive shortening of the alkyl chain takes place.

The biodegradation of LAS is affected by a number of factors, among which are the concentration of dissolved oxygen, aggregation with cationic surfactants, formation of insoluble calcium and magnesium salts, presence of other organic contaminants, and the effect of LAS on the pH during aerobic degradation (Abd-Allah and Srorr, 1998; deWolf and Feijtel, 1998; Fox et al., 1997; García et al., 1996; Krueger et al., 1998; Utsunomiya et al., 1997, 1998). The rates of LAS biodegradation increase with dissolved oxygen concentration, and the longer alkyl chain homologues (C12 and C13) are preferentially biodegraded.

When discussing the further fate of LAS and its degradation product sulfophenyl carboxylate in the coastal environment, it has to be taken into account that the overall metabolic activity of estuarine and marine microbial communities is generally lower compared with that of continental waters. The precipitation of LAS as magnesium and calcium salts might become the principal elimination route because of high concentrations of both ions in these environmental settings. Removal of dissolved LAS from the water phase may likewise occur by sorption onto particulate matter and sediments (Rubio et al., 1996). In the latter compartment, LAS is likely to be accumulated due to the low or null dissolved oxygen content near the bottom.

The highly polar character of sulfophenyl carboxylate, and the lack of a hydrophobic moiety as present in the LAS molecule, which is essential for interaction with the organic matter, largely impedes an accumulation of sulfophenyl carboxylate in sediments. Whereas anionic surfactants have been found at mg/kg levels in riverine and lake sediments, the corresponding degradation products were not detected in any instance (Trehy et al., 1996).

García et al. (2005) studied the sorption of LAS homologues on anaerobic sludge, and determined the distribution of each one between aqueous and solid phases and, consequently, its availability. The surfactant concentration in the liquid phase decreased significantly as the LAS chain length increased, and a linear relationship was found between the partition coefficient and the alkyl chain length. Negligible primary biodegradation of the LAS homologues and isomers was detected in anaerobic conditions. Sulfophenyl carboxylates analysis by LC-MS confirmed the poor transformation of the LAS molecules. However, significant differences on the extent of the biogas production were observed depending on the LAS homologue. Thus, the shortest LAS homologues (C10-LAS and C12-LAS) produced a certain extent

of biogas production inhibition, whereas C14-LAS enhanced production. The inhibition observed for most of the hydrophilic compounds could be related to its higher concentration in the aqueous phase. C14-LAS seems to promote the availability of organic compounds associated with the anaerobic sludge and, consequently, their mineralization.

Sulfophenyl carboxylates present net rates of mineralization in seawater that are comparable to those of compounds utilized as reference in biodegradation assays in seawater (i.e., aniline, sodium benzoate). Thus, they can be considered highly susceptible to mineralization by the microbiota present in seawater (Perales et al., 2003).

Alkylphenol ethoxylates undergo almost complete primary degradation in the presence of oxygen. Although rapid primary degradation takes place, degradation by-products are not as available to microorganisms as the original product. The polyoxyethylene chain appears to be readily biodegradable, but the nonylphenol (NP) derivative seems to be more resistent (Balson and Felix, 1995).

Fatty alcohol sulphates are rapidly degraded under aerobic conditions. Their degradation is thought to involve the enzymatic cleavage of the sulphate ester bonds to give inorganic sulphate and a fatty alcohol. The fatty alcohol is oxidized to an aldehyde and subsequently to a fatty acid with further oxidation via the beta-oxidation pathway. Fatty alcohol sulphates and their degradation products are ultimately biodegradable (Bruce et al., 1966; Thomas and White, 1989).

3.2 Transport and Fate of Surfactants in Wastewater Treatment Plants

LAS (Table 2) are the most important anionic surfactants that will reach the municipal WWTP nearly unchanged. An extensive body of studies conducted on the fate of LAS during wastewater treatment has indicated that they are efficiently removed by physical, chemical, and biological processes. Apart from precipitation and adsorption onto suspended solids, which can range from 30 to 70% (Berna et al., 1989) of the initial contents, microbial degradation generally accounts for the major elimination route—typically around 80%—resulting in an overall reduction of 95–99.5% of the LAS load in activated sludge systems (Painter and Zabel, 1989). Nonetheless, some residues of the intact surfactant, together with its aerobic breakdown intermediates, sulfophenyl carboxylates (Fig. 3), enter the receiving waters via treatment plant outlets. In spite of the enormous amounts of LAS used, concentrations in surface waters are found in the lower μg/L range (Schöberl, 1995; Tabor and Barber, 1996).

In contrast to this, if domestic wastewater is discharged directly into natural water streams because of deficient treatment facilities, the surfactant levels in water can be considerably higher. While the majority of the households are connected to treatment plants in Western Europe and the United States, the emission of untreated sewage into rivers is still widely practiced in many countries (Eichhorn et al., 2002; Ojeda and Fernández-Cirelli, 2008).

This causes particular concern since, under these circumstances, aquatic organisms are exposed to considerable levels of surfactants that exhibit relatively high toxicities (Schöberl, 1997).

In wastewater, the extent of LAS adsorption to particulate matter has been shown to be dependent upon a number of factors—the most significant being the type of LAS homologue present. The longer alkyl chains confer greater hydrophobicity, thus increasing adsorptive tendency. For each carbon atom added to the alkyl chain, a two- to three-fold increase in the Ka (association constant) for LAS was observed. The chemical composition of the effluent may have a significant effect upon the adsorption of LAS. Water hardness could significantly alter partition coefficients of LAS in raw wastewater. Waters high in Ca concentrations yielded sludge from primary settling tanks that contained 30–35% of the LAS concentration of the raw sewage, but relatively soft water yielded only 10–20% (Berna et al., 1991). A significant proportion of LAS in raw wastewater (10–35%) adsorbs to particulate matter. Sediment removed from primary settling tanks is relatively rich in LAS, with concentrations ranging from 5–15 g/L (Brunner et al., 1988; de Henau et al., 1986; McEvoy and Giger, 1985).

The presence of high concentrations of LAS in sewage sludge leaving the treatment plant is dependent upon the type of treatment the sludge undergoes. As stated before, LAS are readily degradable under aerobic conditions, since the alkyl chain oxidation at the terminal methyl group requires the presence of molecular oxygen (Fig. 3).

The outcome of pilot surfactant monitoring studies at activated sludge of treatment plants in five European countries, using LAS as the reference compound, has been reported. A very high average LAS removal from water of 99.2% has been found during aerobic wastewater treatment. Hence, only low concentrations (0.009–0.140 mg/L) of LAS were discharged to the receiving waters, well below the predicted no effect concentrations (100–350 μg/L for aquatic ecosystems). The concentrations of LAS found on sediments at river-sampling sites below the effluent discharges were also low, ranging from 0.49 to 5.3 μg/g (Waters and Feijtei, 1995). Transport of surfactants in treatment plants is shown as an example in Fig. 4 (Scott and Jones, 2000). Figures may vary from one plant to another.

Aerobically digested sewage sludge presents LAS concentrations of 100–500 mg/kg dry weight—considerably lower than those found in anaerobically treated sludge (5,000–15,000 mg/kg dry weight). Therefore, the extent of LAS contamination of sewage sludge is greatly dependent upon the individual treatment plant and the employed method of sludge digestion.

Batch anaerobic biodegradation tests at laboratory scale with different LAS at increasing concentrations were performed to investigate the effect of LAS homologues on the anaerobic digestion process of sewage sludge. Addition of LAS homologues to the anaerobic digesters increased the biogas production at surfactant concentrations of 5–10 g/kg dry sludge, and gave rise to a partial or total inhibition of the methanogenic activity at higher surfactant loads. Therefore, at the usual LAS concentration ranges in sewage sludge, no adverse effects on the anaerobic digesters in a WWTP can be expected. The increase of biogas production at low

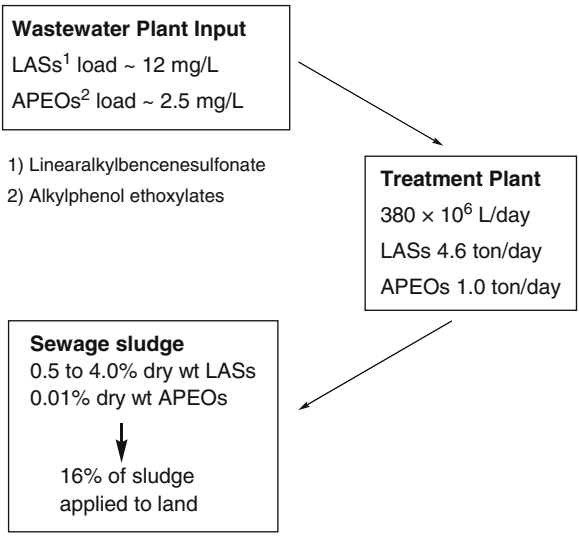


Fig. 4 Transport of surfactants through a wastewater treatment plant

surfactant concentrations was attributed to an increase of the bioavailability and subsequent biodegradation of organic pollutants associated with the sludge, promoted by the surfactant adsorption at the solid/liquid interface (Garcia et al., 2006).

In accordance with the results described above, it is evident that LAS, cationic surfactants, alkylphenol ethoxylates, and alcohol ethoxylates are all relatively resistant to degradation in anaerobic environments. Because anaerobic digestion is the predominant treatment of sludge from primary and secondary settling tanks, and because the amphiphilic nature of surfactants promotes their adsorption to particle surfaces in sewage, it appears that surfactants pass through a treatment plant relatively untreated. Application of sludge to agricultural land may be a large source of surfactants in the soil environment. However, it appears that once reintroduced into an aerobic environment, such as the soil, the surfactants are rapidly degraded. Anionic and cationic surfactants are readily biodegradable in aerobic environments, but the last group is toxic even at low concentrations. Therefore, application to agricultural soil may have detrimental effect to the soil biota. Alkylphenol ethoxylates molecules are readily degradable aerobically, however NP (one of its primary degradation products) has been described as an estrogenic compound active in the environment. Nonylphenol has a strong affinity for soil particles and is less biodegradable than alkylphenol ethoxylates. At present, the authors can find scarce literature published concerning the estrogen-mimicking properties of NP in sludge-amended soils, and this is a field that needs further research.

3.3 Fate of Surfactants in Waters and Soils

Surfactants enter the environment through treated wastewater discharge into surface waters, pesticide application, sludge disposal on land, or other activities. Once in the environment, they undergo processes such as sorption onto soil or water particles and degradation. Knowledge of the processes involved in the distribution of these surfactants among ecosystem compartments is essential to understanding their behavior in the environment. Sorption of a surfactant on a sediment or soil depends on many factors, including their physicochemical properties, sediment/soil nature, and environmental parameters. Sorption on sludge, sediment, and soils of surfactants is relatively high, and their order of sorption is: cationic > non-ionic > anionic. Cationic surfactants with a positive charge have a strong affinity for the surface of particulates in sewage sludge, which are predominantly negatively charged (Topping and Waters, 1982).

The presence of LAS and sulfophenyl carboxylates was investigated in a river located northeast of the city of Niteroi (State of Rio de Janeiro, Brazil) by monitoring the concentrations of both compounds at several stations along the river course (Eichhorn et al., 2002). This river, receiving discharges of untreated domestic wastewater from several villages with populations amounting to about 20,000 inhabitants, contained considerable amounts of LAS from 12 to 155 μg/L, as well as its metabolite sulfophenyl carboxylates from 1.7 to 12 μg/L. The findings show that microbial communities present in the river are sufficient to oxidize LAS, yielding long-chain sulfophenyl carboxylates.

The impact of raw wastewater discharges of municipal and industrial origin on surface water was studied in a Taiwanese river, identifying (apart from LAS and sulfophenyl carboxylates) nonionic surfactants and their metabolites (Ding et al., 1998). Concentrations of the surfactants ranged between 11.7 and 135 μg/L, while the degradation products were found from 0.3 to 3.1 μg/L.

Trehy et al. (1996) reported on levels of LAS and sulfophenyl carboxylates in U.S. receiving waters upstream and downstream of domestic treatment plants. The values averaged 16 and 35 μg/L for LAS, while the mean concentration of sulfophenyl carboxylates amounted to 9.3 and 31 μg/L, respectively. A monitoring study performed in Italy comprised two strongly polluted riverine sites sampled upstream and downstream of a treatment plant (Marcomini et al., 2000). The average concentration of LAS was slightly increasing from 177 to 187 μg/L, whereas the sulfophenyl carboxylates level ranged from 368 to 420 μg/L. From these data describing distinct wastewater disposal situations, the ratio of LAS to sulfophenyl carboxylates may be indicative of the emitted treated wastewater. A low value of the ratio as found in the U.S. (0.9) and Italian (0.2) work may be indicative of wastewater treatment (Trehy et al., 1996; Marcomini et al., 2000). Elevated values as observed in the Taiwanese study ranging from 270 to 6.7, and also in the Brazilian rivers between 13 and 1.6, may be indicative of a high percentage of untreated wastewater (Ding et al., 1998; Eichhorn et al., 2002).

In the Brazilian river, a rapid decrease in concentrations of LAS in the water has been observed 1.5 km downstream from the discharge point, particularly when the

flow in the river is high. The decrease in concentration is related to both biodegradation and the loss of surfactants due to adsorption on river sediments and suspended solids in the raw sewage (Eichhorn et al., 2002).

As for the fate of surfactants after environmental discharge of untreated wastewater, the concentration in the water might be reduced by sorption onto riverine sediments, as well as by biodegradation through endogenous bacterial communities present in the stream, with slower kinetics compared to treatment plant (Eichhorn et al., 2002). The high water solubility of LAS, and of their even more polar metabolites, enables their convectional transport over relatively long distances. Ultimately, the mouthing of polluted rivers into estuaries and subsequently into the sea contributes to the contamination of coastal waters.

The fate and effects of LAS in the aquatic environment have been studied extensively, whereas the terrestrial environment has received considerably less attention. Soil is exposed to a considerable quantity of surfactants, and even at low concentrations, surfactants seem to significantly alter soil physics, soil chemistry, and soil biology, with sorption processes playing a dominant role. The literature concerning the fate of surfactants in wastewater sludge-amended soil is heavily biased towards the study of LAS, with other surfactants receiving little or no attention.

LAS was monitored in sludge-amended soils in a Spanish grapevine farm and a vegetable farm. From relatively high sludge application concentrations of 7,000–30,200 mg/kg dry weight, initial soil concentrations of 16 and 53 mg/kg, respectively, were observed. After periods of 90 and 170 days, the soil concentrations of LAS were 0.3 mg/kg. After an initial period of LAS removal, soil concentrations appeared to level out and did not decrease further, suggesting that LAS may be incorporated into the soil particles and/or be associated with the soil organic matter. This fact renders the surfactant unavailable to the microorganisms responsible for their biodegradation (Berna et al., 1989).

The concentration of surfactants in soils that have not received sludge recently is generally less than 1 mg LAS/kg and not more than 5 mg LAS/kg. This is below the lowest concentration of LAS for which effects have been observed in the laboratory. The laboratory data are in accordance with field studies using aqueous solutions of LAS (sodium salt). However, observations on the ecological impact of sewage sludge applications, or the application of LAS spiked into sludge, indicates a lower toxicity of LAS when applied *via* sludge. Jensen (1999) concluded that LAS can be found in high concentrations in sewage sludge, but that the relatively rapid aerobic degradation and the reduced bioavailability when applied *via* sludge will most likely prevent LAS from posing a threat to terrestrial ecosystems on a long-term basis.

Once LAS is removed from the anaerobic environment of sludge digestion and/or storage, aerobic bacteria begin to metabolize these surfactants. Rapid metabolism leads to relatively short LAS half-lives. Most authors who have carried out the monitoring of LAS residence in sludge-amended soils agree that due to their relatively high biodegradability in the aerobic environment, there is little chance of accumulation of LAS in soil.

Experimental measurements of the adsorption of LAS on soils were made at 25°C using a continuous adsorption apparatus. The adsorption of LAS on natural soils could be divided into two stages: linear and exponentially increasing isotherms. At low LAS concentrations (<90 μg/mL), the adsorption isotherms were linear and Kd was among 1.2–2.0. At high LAS levels (>90 μg/mL), cooperative adsorption was observed and the amount of LAS adsorbed increased exponentially with the increase of LAS concentration in the solution. LAS adsorption mechanisms on soil are mainly specific-site surface interactions and hydrogen bonding. The LAS adsorption capacity of a soil significantly depends on its clay content. Under real soil environments where LAS levels are rather low, the LAS adsorption ability of a soil is very weak (Ou et al., 1996).

To investigate the behavior of surfactants in soil ecosystems, the sorption of LAS on soils from three different areas of Northern Greece, and with different organic matter content, was studied. LAS sorption on these soils decreased with increasing pH, and correlated positively with the organic matter content of the soils (Fvtianos et al., 1998). The pH value controls the degree of sorption and desorption processes, the solubility and the activity of potentially degrading microorganisms. As far as anionic surfactants are concerned, increasing adsorption has been reported with decreasing pH values, due to a higher positive charge of colloidal surfaces.

It has been shown that the saturated adsorption amount of LAS on soils was lower compared with the alcohol ethoxylates. Adsorption of anionic surfactants decreased in the presence of nonionic surfactants. These could result from: (i) the difference between the critical micelle concentration of mixed surfactants at different molar ratios; (ii) the hydrocarbon chain–chain interactions between LAS and alcohol ethoxylates; (iii) or the saturation of a majority of adsorption sites by alcohol ethoxylates. The adsorption of both surfactants on soils decreased with the increase of pH in mixed surfactant solutions, as well as with a decrease in ion strength (Rao and Re, 2006).

On the other hand, fatty alcohol sulphates appear to be readily bioavailable by microorganisms under both aerobic and anaerobic conditions, and easily degradable both primarily and ultimately. Therefore, treatment in an aerobic treatment plant is entirely sufficient to eliminate fatty alcohol sulphates, and little possibility exists for these surfactants to reach the soil environment via sludge amendment. Lauryl ether sulfate is readily bioavailable in both aerobic and anaerobic environments, with comparable primary and ultimate degradation rates for fatty alcohol sulphates under aerobic conditions (Fischer, 1982; Schöberl et al., 1988).

The effect of surfactant on plant growth from the use of sewage sludge in soils is difficult to assess because sludge generally promotes plant growth. Adverse effects on plant growth were observed at 392 μg/L, but long-term monitoring of 46 environmental sites gave 63 μg/L of LAS—far from the concentration reported to have adverse effects (Scott and Jones, 2000). Nonetheless, a study of the fate of LAS and other xenobiotics in a sandy soil after sludge spreading on a 30-year field-scale record experiment have been done to evaluate the real impact of the presence of LAS and others compounds in the soil. After 12 years since the last addition of sewage sludge, the residual concentrations of most xenobiotics remain from 2 to 10

times higher than the content of the control soil, even though these levels are inferior to the Predicted Noneffect Concentration (PNEC). Only LAS levels went back to the level in the control soil. However, only the LAS concentration is above the PNEC during all the experiments due to the very high level of LAS in the sludge (20 g/kg dry weight). These results show that even though this compound is much more degradable than the rest of the evaluated xenobiotic, it may have a long-term effect in the soil if high quantities are spread (Patureau et al., 2007).

It appears that surfactant application in suitable concentrations to aerobic soils is quite safe due to rapid biodegradation rates. However, the temptation to dispose of sludge on nonagricultural soils should be carefully investigated. Soils that are anaerobic may not be appropriate sites for amendment. Such soils may exhibit accumulation of surfactants as biodegradation is retarded and may ultimately result in surfactant contamination of the environment.

3.4 Interaction of Surfactants with Soil Contaminants

As stated, surfactants may form micelles in solution due to the presence of both hydrophobic and hydrophilic groups in each surfactant molecule. The organic interior of micelles acts as an organic pseudophase into which organic contaminants can be partitioned. This phenomenon can greatly enhance the total concentration of the contaminant in solution above its aqueous solubility limit if surfactants are present. In fact, the solubility of a hydrophobic solute in surfactant micelles has been found to be several orders of magnitude larger than its aqueous solubility in the absence of surfactants. The extent to which a solute will concentrate in a micelle can be related to the octanol-water partition coefficient (Kow) of the solute. In general, the larger the Kow of a solute, the greater is its tendency to concentrate inside the micelle. There are two mechanisms by which surfactants can interact with organic compounds in soils. The first and most important mechanism involves solubilization of contaminants in surfactant micelles. The second mechanism involves the mobilization of contaminants from the soil; this depends on the tendency of surfactants to reduce the interfacial tensions and capillary forces trapping the contaminant in the soil. These interactions affect the bioavailability of soil pollutants, and therefore can be used for soil bioremediation (Haigh, 1996).

The use of surfactants to decontaminate ground water aquifers and in soil clean-up operations is well-established, and both anionic and nonionic surfactants have been used to remediate land polluted with oils and hydrocarbons as well. Both anionic and nonionic surfactants have been used to remediate land polluted with oils and hydrocarbons, as well as with many other organic contaminants.

Surfactant addition has been investigated as an innovative technique for decreasing interfacial tension between the soil's nonaqueous phase (NAP) and water, and for enhancing aqueous-phase solubility. The NAP contaminants can be solubilized through incorporation of contaminant molecules into micelles of surfactants. Water cannot be recommended for an efficient removal of pollutants from a contaminated

soil, and thus organic surfactants should be relied upon in soil washing procedures (Santharam et al., 1997).

Two synthetic surfactants—sodium dodecylsulphate (SDS) and Triton X-100 (TX100)—and a solution of a natural surfactant a humic acid-HA at its CMC were used for soil depollution. Soil A was richer in polycyclic aromatic hydrocarbons, whereas soil B had a larger content of thiophenes. The synthetic surfactant mixture that was used was able to reduce the content of contaminants from 80 to more than 90%in both soils. Natural nontoxic surfactants such as HA removed similar amounts of contaminants from a polluted soil as the synthetic surfactants did. However, synthetic surfactants that are efficient in soil washing may become a further environmental problem because of their toxicity. In conclusion, a natural surfactant, such as a humic acid solution, can be used for washing a contaminated soil with the same efficiency and less toxicity as that of synthetic surfactants to avoid further environmental problems (Conte et al., 2005).

The potential effects of selected surfactants on the biodegradation of chlorinated hydrocarbons in wastewater has also been investigated. Biodegradation of a real waste containing a broad array of hazardous contaminants was significantly enhanced by the amendment of mineral nutrients and surfactants. Contaminants included hexachlorobutadiene (HCBD), hexachlorobenzene (HCB), trichloroethylene (TCE), halogenated organic solvents (1,2-dichloroethane (DCE), tetrachloroethane), volatile aromatic hydrocarbons including benzene and toluene, and polynuclear aromatic hydrocarbons (PAH). The reduction of contaminants was 49% higher for the mixture of wastewater with surfactants. Both a nonionic surfactant and sodium dodecylsulphate have been assayed (Zhang et al., 1998).

Soils contaminated with both heavy metals and hydrophobic organic contaminants are commonly found. Ethylenediaminetetraacetic acid (EDTA)- and sodium dodecylsulphate-enhanced washing was studied for remediation of Pb- and/or marine diesel fuel-contaminated soils. The feasibility of recovery and reuse of ethylenediaminetetraacetic acid and sodium dodecylsulphate, as well as the physicochemical interactions among the chemical agents, contaminants, and soils were extensively investigated using batch experiments. The optimal washing sequence was then determined. The experimental results showed that EDTA could be recovered and reused for four cycles without significant loss of its chelating capacity, while the extraction capability of SDS was noticeably reduced after each reuse cycle. The free phase of marine diesel fuel in soils physically isolated the sorbed Pb on soils, thus reducing its extraction by ethylenediaminetetraacetic acid. The presence of sodium dodecylsulphate alone, or together with low ethylenediaminetetraacetic acid concentration was found to enhance Pb removal, probably via electrostatic interaction and dissolution of soil organic matter (Zhang et al., 2007).

In addition to soil cleaning properties, some surfactants, even at very low concentrations, have been shown to enhance the biodegradation of certain xenobiotics in soil. However, at higher surfactant concentrations, it has been reported that degradation can be delayed due to the partitioning of xenobiotics into surfactant micelles. Surfactant-pollutant interactions in soil are very complex and depend heavily on a

range of parameters, including surfactant concentration in soil-water compared with CMC, adsorption characteristics of the surfactant and pollutant, solubility of the pollutant, and soil type. The most important parameter in terms of the ability of a surfactant to mobilize hydrophobic xenobiotics in contaminated soil is the surfactant CMC. In general, concentrations of surfactant in soil-water below the CMC have little or no effect on solubilization of hydrophobic materials. Only when micelles are present does significant desorption of such pollutants from soil surfaces occur. Conversely, under some conditions, and usually at concentrations well below the CMC, the presence of surfactant can enhance the adsorption of hydrophobic xenobiotics to soil particles (Haigh, 1996). This fact has been attributed to partitioning of the xenobiotic into surfactant hemimicelles formed on the soil surface. In environments such as soils and sediments, adsorption of surfactants to surfaces results in much higher total surfactant concentrations being necessary to achieve micellization in porewater than would be necessary in clean water systems. Therefore, much higher concentrations of surfactant are required than might be expected to cause significant changes in xenobiotic behavior. Such high concentrations are not typical of those found in sludge-amended soil.

4 Conclusion

Detergents are widely used, not only domestically but in many different industries such as cosmetics, personal care, household products, paint, coatings, textiles, dyes, polymers, food, agrochemical supplies, and oil. The formulations include all types of surfactants—anionic, cationic, nonionic and amphoteric—but the most common surfactant by far is LAS, which is an anionic surfactant. Surfactants can reach agricultural soils different ways, with the most relevant ones being soil amendment by sewage sludge and pesticides applications on the crops. Depending on the type of surfactants, they can be degraded in aerobic or anaerobic conditions producing different types of metabolites. From the literature data, it is evident that LAS, cationic surfactants, and alkylphenol ethoxylates are all relatively resistant to degradation in anaerobic conditions. Therefore, the application of sludge to agricultural soils could be a large source of surfactants if sludge digestion has been performed anaerobically. However, when the compounds are reintroduced into an aerobic environment, such as soil, they are rapidly degraded. More studies regarding cationic surfactants degradation are needed to evaluate their potential toxicity in the environment due to microorganism inhibitory activity.

The interaction between surfactants and pollutants is an important subject of study nowadays, because surfactants can be used in bioremediation of soils, due to their capacity to affect the bioavailability of contaminants such as heavy metals and organic compounds. Finally, surfactant input in agricultural soils is not negligible and is increasing worldwide. Therefore, knowledge on their introduction pathway, their interaction with polar and nonpolar contaminants, and their fate and persistence in the environment is required to evaluate their behavior in agricultural areas under the increasing use of organic amendments and agrochemicals.

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Mineral Nutrition for Legume-Rhizobia Symbiosis: B, Ca, N, P, S, K, Fe, Mo, Co, and Ni: A Review

Ildefonso Bonilla and Luis Bolaños

Abstract The intensification and expansion of modern agriculture starting in the middle of the 20th century accounted for a substantial increase in crop yield. However, productivity growth has led to an extraordinary simplification of farming systems and greater reliance on external inputs. The extensive use of pesticides and fertilizers are the cause of frequent health problems and pollution of natural ecosystems. Such evidence has led to debate about the sustainability of current intensive agricultural practices. Organic farming, which aims to produce healthy food and to respect the environment, emerges as an alternative to the negative consequences of conventional farming. In the context of sustainable organic agriculture, the successful use of biological nitrogen fixation without a decrease in productivity will reduce chemical fertilization. For that, it is important to have previous knowledge of mineral nutrient requirements to optimize symbiotic nitrogen fixation and legume crop production. Here, we first review the basic concepts of mineral nutrition, as well as the importance of mineral nutrients specifically for biological nitrogen fixation in the legume-rhizobia symbiosis. Second, a broad summary of the roles of boron and calcium in plants, with special attention to their key functions in nitrogen fixation and legume-rhizobia symbiosis, will be the central topic of this review. Symbiotic nitrogen fixation is an optimal alternative to reducing the application of chemical N-fertilizer, but demand for some nutrients is higher for legume nodule development and function than for non-nodulated legumes, and corrections of nutrient deficiencies are sometimes needed to ensure crop success. Phosphorus is a common limiting nutrient of nodulated legume growth, because of phosphate requirements for nodulation and for the very energy-expensive nitrogen fixation reaction. The enhancement of the association of nodulated-legumes with vesicular-arbuscular mycorhizas, improving phosphorus uptake, is an ecological and cheap way to correct P limitation. Sulphur and potassium are not usually limiting nutrients for nodulated legumes, although a K^+ supplement for osmoadaptation has to

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be considered for growth in saline soils. Similarly, although demand for cobalt or nickel is higher with nodulated than with non-nodulated legumes, the soil limitations of these micronutrients are unclear. Conversely, iron and molybdenum limitations for nodulated legumes are common, even in soil with sufficient Fe and Mo, because of the anaerobic and acidic environment inside the nodule that limits the availability of these micronutrients. Therefore, Fe and Mo fertilization cannot be ruled out in sustainable agriculture based on nodulated legumes. Among mineral nutrients, B and Ca are undoubtedly the nutrients with a major effect on legume symbiosis. Both nodulation and nitrogen fixation depend on B and Ca^{2+}, with calcium more necessary for early symbiotic events and B for nodule maturation. Boron deficiency is very common, and there is a risk of toxicity following B fertilization because it appears at concentrations close to sufficiency; and, in boron-deficient soils, an early small supplement of calcium prevents the effects of B limitation during nodulation. Therefore, a proper B–Ca feeding will greatly correct boron deficiency and improve crop production. Overall, improvement of symbiotic nitrogen fixation in legumes, combined with mycorhizal associations, is a natural fertilizing alternative to conventional chemical fertilizers. Nevertheless, small and controlled application of conventional farming practices has to be considered to correct nutrient limitations, increase crop production, and satisfy the high demand of agriculturally derived food.

Keywords Boron · Calcium · Cobalt · Iron · Legume-symbiosis · Mineral nutrition · Molybdenum · Nickel · Nitrogen fixation · Nodule development · Phosphorus · Potassium · Sustainable agriculture

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1 Introduction

The need to satisfy nutritional demands on behalf of the world's current population is leading to the exponential increase of agricultural production. Such a high demand has been translated into a massive and sometimes indiscriminate application of

pesticides and nitrogen fertilizers as the unique human response to the lack of nitrogen available for plants in cultivable soils (Peoples et al., 1995). Since the middle of the 20th century, world chemical fertilizer consumption has increased dramatically. Global fertilizer consumption in 2000 was 136 million (Lal, 2004). Fertilizer use has leveled off in highly populated countries such as the United States (about 19 million tons per year since 1984) and India (16 million tons per year since 1998). China is now the top consumer of chemical fertilizers, with more than 40 million tons in 2004. European Union statistics point to excessive nitrogen fertilization (55 kg ha^{-1} per year) and application of active pesticides (2.0 kg ha^{-1} in 2001) (Eurostat, 2006). Massive application of fertilizers and pesticides affects both farmers and residents of rural areas and creates pollution of natural ecosystems associated with agriculture (Liebman, 2001; Matson et al., 1997). Serious problems derived from these practices have led to a call for sustainable agriculture based on organic ecological farming, which produces healthy food. The high costs of increasing the production of fertilizers for this practice are undoubtedly a problem at the economic level; and increased production has also been translated into another serious constraint, not only for humans, but for the survival of the biosphere: the contamination of continental waters by increasing nitrate contents until toxic levels cause the consequent eutrophication of lakes and rivers. For example, in the United States, the estimated environmental and health care costs of the recommended use of pesticides are about $10 billion per year—the excessive fertilizer use costs $2.5 billion from wasted fertilizer inputs, and the costs of public and environmental health issues related to soil erosion by conventional modern agriculture exceed $45 billion yearly (Pimentel, 2005; Pimentel et. al., 1995).

The use of N as a fertilizer has degraded huge land extensions around the world and biological nitrogen fixation as an alternative to chemical fertilization is required to replace tons of fertilizers (Burris, 1994). This natural way of supplying nitrogen for plants is due to the capacity of certain soil microorganisms to fix atmospheric N_2 and to transform it into ammonium, which can be used by the plant when the fixing microorganism establishes a symbiotic relationship with it. Biological nitrogen fixation has attracted great agronomic interest. It is estimated that rhizobial symbiosis, with over one hundred agriculturally important legumes, accounts for at least half of the annual amount of nitrogen fixation in soil ecosystems (Peoples and Craswell, 1992). This plant–microorganism symbiosis offers a series of advantages over N fertilizer, among which are the high efficiency in the utilization of N by the plant, sometimes near 100%; the minimization of leaching of nitrogenous fertilizers to the soil; and the reduction of soil and water contamination.

All living N_2-fixers are prokaryotes that do not share a homogenous taxonomic group; the only characteristic they share is the presence of the nitrogenase enzyme complex (for a review, see Sprent and Sprent, 1990). They include phototrophic organisms like bacteria of the families Rhodospirillaceae, Chlorobiaceae, and Cyanobacteriae; chemoautotrophs like *Thiobacillus, Xanthobacter,* and *Desulfovibrio*; heterotrophs like *Azotobacter, Enterobacter, Klebsiella,* and *Clostridium;* and bacteria of the Frankiaceae and Rhizobiaceae families. These organisms can fix nitrogen as free-living forms (with the exception of the Rhizobiaceae), or by

establishing symbiotic relationships with other organisms. The most extended N_2-fixing symbioses involving higher plants are those established between rhizobia and legumes and by *Frankia* with actinorhizal plants. Actinorhiza pioneers plants in devastated soils; *Frankia* symbioses acquire great environmental importance for the recovery of eroded soils (Tate, 1995). Meanwhile, rhizobia-legume symbioses have an enormous potential to produce food for humans (grain legumes) (Wani et al., 1995) or animals (pasture) (Thomas, 1995); to renew cultivable soils by the practice of culture rotation; and definitely to reduce the use of chemical fertilizers.

Since the classic studies of Hellriegel and Wilfarth (1888), clearly establishing that microbes inside the root nodules allowed legumes to obtain N from the air, the interaction between rhizobia and legumes has been widely studied, but some aspects are still unknown. One of them is the influence of various nutrients required by the system both during the establishment or/and development of the symbiosis and during nodule organogenesis.

2 Mineral Nutrition in the Legume-Rhizobia Nitrogen Fixing Symbiosis

After more than one century of research, great knowledge about molecular aspects of the legume-rhizobia interaction has been acquired. Nevertheless, several shadowy aspects of physiological, environmental, and nutritional subjects affecting one or both symbiotic partners, or specifically their interaction, still remain to be elucidated before we fully understand the symbiotic process.

In general, plant development depends on several genetic and environmental factors. Considering a plant in a concrete environment, the more important factors for growth are light, water, CO_2, and nutrients. Atmospheric CO_2 and soil water contribute C, O, and H that makes up about 90–95% of the plant's dry weight. The remaining 5–10% (N, P, K, S, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, Cl, Ni, plus Na or Si in some plant species) is called the mineral fraction. Despite their low, or even very low, quantitative presence, all of these mineral nutrients are absolutely essential for processes related to plant growth and development, including plant-microbe interactions like those resulting in legume-rhizobia symbiosis. For example, Mo is required at a concentration of only 0.1 ppm (part per million, mg $kg^{-}1$ dry weight), but it is absolutely essential. Several plants can tolerate and accumulate heavy metals at concentrations unusually high (Memon et al., 2001), but heavy metals are not essential nutrients for those plants. Consequently, it has to be clearly stated that neither the presence nor the concentration of a mineral element are valid criteria of essentiality. In the absence of a given mineral, only the fact that a plant is unable to complete its vital cycle can grant the category of essential nutrient to that mineral. To learn about plant mineral nutrition, Marschner (1995) and Epstein and Bloom (2005) are excellent monographic books.

It is important to emphasize that nutrient deficiencies can affect not only plants but also rhizobia soil populations (reviewed by O'Hara, 2001). A considerable variability of response among genus, species, and strains makes nutritional effects on bacteria highly unknown. Furthermore, competition between plants and soil microorganisms for nutrients can induce deficiencies in rhizobia, especially of those nutrients with low availability in many soils, like phosphorus or iron, affecting the nodulation capacity. Besides the effects on both symbiotic partners, some nutrients can directly play a specific role in some stages of the symbiosis development. Finally, nutrient balance can modify the absorption and accumulation of other mineral nutrients, affecting the growth of both symbionts, and the regulation of gene expression that governs the interaction. Therefore, integrated approaches involving plant physiology, microbiology, and molecular biology studies are required to fully understand nutritional stresses in the legume-rhizobia symbiosis. Meanwhile, this review will be focused on mineral nutrients with a strong effect on the symbiotic process, rather than on the growth of free-living bacteria and plants.

Although any one of the above listed 17 nutrients considered essential for all plants is also essential for legume-rhizobia symbiosis, some of them play particular roles during the symbiotic interaction. Of course, N has to be highlighted as the element that has to be fixed from atmospheric N_2 by bacterial nitrogenase. In addition, other mineral nutrients with a more specific effect on the interaction, including elements like Co or Ni, either required for the microsimbiont or exclusively for the N_2-fixing event, are briefly reviewed herein. Furthermore, B and Ca are two nutrients described as highly in demand for nodulated plants; both of them have strong effects on nodulation and nitrogen fixation (Redondo-Nieto et al., 2003), and a relationship between B and Ca in many physiological plant processes was early stated (Reeve and Shive, 1944). Therefore the roles of both mineral nutrients in symbiotic nitrogen fixation will be more profusely described.

As stated above, deficiencies of nutrients herein reviewed, especially B and Ca, affect the symbiotic process, and hence crop production. The legume-rhizobia symbiosis is a highly regulated process of organogenesis, and different mineral nutrients have strong effects on different developmental stages of the symbiotic interaction, and/or on the nitrogen fixation process itself. Knowledge of the roles of mineral nutrients is useful in order to establish the right nutrient supplements to improve crop legume production without affecting ecological farming practices.

2.1 Macronutrients

2.1.1 Nitrogen

The symbiosis between soil rhizobia and legumes is not obligatory. In soils with enough available N, both bacteria and plants may remain unassociated during their full life cycle. However, N deficiency triggers the interaction, and only while low

N conditions are maintained does symbiosis develop successfully into a nodule where atmospheric molecular N_2 is reduced. Depending on the moment of application, the supply of combined nitrogen reduces nodulation by reduction of bacterial adsorption to the roots (Munns, 1968) or by diminution of infection (Abdel-Wahab and Abd-Alla, 1995); inhibits leghemoglobin synthesis (Bisseling et al., 1978) and nitrogenase activity; and accelerates nodule senescence (Becana and Sprent, 1987). Maximum N_2-fixation to satisfy N needs of a plant requires that the legume be adequately nodulated, therefore optimization of rhizobial infection in a context of sustainable agriculture demands a reduction of fertilizer-N application.

2.1.2 Phosphorus, Sulfur, and Potassium

Among macronutrients, along with calcium (to be treated later on), P is a common limiting nutrient of N_2-fixing legume crop production in many areas (Pereira and Bliss, 1989). Phosphate acts on nodulation and nitrogen fixation (Ssali and Keya, 1983). In energy terms, nitrogen fixation is a very expensive process, exceeding 16–18 mol ATP per mol N_2 fixed (Bergersen, 1991); consequently, N_2-fixing legumes will require more P than those supplied with combined nitrogen (Cassman et al., 1981a, b). In a context of sustainable agriculture, infection with vesicular-arbuscular mycorhizas can greatly improve phosphorus uptake (Bucher, 2007) to satisfy the high demand of nodulating legumes, especially in soils with low P availability.

The effects of sulphur and potassium are usually less dramatic, although symbiotic systems have been described as more sensitive to low K than the legumes themselves (Sangakkara et al., 1996). However, in saline soils K^+ acquires great importance as an osmolyte for adaptation (Zahran, 1999). Taking into account that nearly 50% of the world's irrigated land is categorized as having potential salinity problems (Rhoades and Loveday, 1990), a supplement of K^+ has to be considered in order to successfully cultivate symbiotic legumes in saline soils.

2.2 Micronutrients

2.2.1 Iron, Molybdenum

These two nutrients are especially required for nodules. The nitrogenase enzyme system consists of two components: component I is a MoFe protein and component II is Fe-protein. Moreover, other proteins requiring Fe as a cofactor important for symbiotic N_2 fixation are abundant inside the nodules; among them, heme-containing proteins like the oxygen carrier leghemoglobin and the cytochromes or Fe-S proteins such as ferredoxin. Therefore, a particularly high requirement of Fe exists in legume nodules (O'Hara et al., 1988), and low iron availability in soils will affect more nodulated than combined N-fed legumes. Moreover, the anaerobiosis required for N_2-fixation could impair the process of reduction of Fe^{3+} to Fe^{2+}, aggravating a possible Fe limitation (Romera et al., 2004). Similarly, the supply of Mo to soils with low availability of this micronutrient has to be increased for the

development of N_2-fixing legumes. Molybdenum limitation occurs in some naturally acidic, poorly buffered soils. The acidifying effect of nitrogenase activity can also reduce Mo availability, affecting crop legume production (Doerge et al., 1985). Therefore, despite the advantages of improved biological nitrogen fixation for sustainable agriculture, a risk of iron or molybdenum deficiencies has to be considered in symbiotic legumes once the nodule is developed and the nitrogen fixation process takes place.

2.2.2 Cobalt

Cobalt deficiency affects nodule development and function at different levels (Dilworth et al., 1979). The requirement of Co for N_2-fixing nodules was reported by Ahmed and Evans (1960). The dependence of Co, the cobalamin coenzyme B_{12} content, leghemoglobin, and N_2-fixation was later demonstrated (Kliewer and Evans, 1963). Cobalamin (vitamin B_{12}), which has Co(III) as the metal component, is required for enzymes such as methionine syntase; ribonucleotide reductase, involved in bacteroid differentiation (Dilworth and Bisseling, 1984); methylmalonil-coenzyme A mutase, involved in the synthesis of heme groups; and leghemoglobin or bacterial cytochromes (Riley and Dilworth, 1985). Therefore, Co is one of the micronutrients with a strong effect not only during nodule development but also during nodule function.

2.2.3 Nickel

About 200 μg Ni are enough to fully satisfy plant demands, forming complexes with several enzymes (Dalton et al., 1988). Therefore, there is no clear evidence of Ni deficiency in soils, although beneficial effects of Ni supply to plants fed with urea in calcareous soil have been reported (Singh et al., 1990). Both plant and rhizobial ureases are Ni-requiring enzymes. In legumes like bean or soybean, developing determinate nodules, ureides are the dominant form of transport from nodules to shoots of fixed nitrogen (Atkins, 1987). Urea is an intermediate of nitrogen and ureide metabolism, and has to be degraded by urease. Otherwise, accumulation of urea will lead to leaf necrosis (Krogmeier et al., 1991). Besides urease, rhizobial hydrogenase also requires Ni. These enzymes recycle hydrogen generated by nitrogenase enzymes, increasing the efficiency of the nitrogen fixation process (Maier and Triplett, 1996). A low level of Ni in agricultural soils limits hydrogenase activity (Ureta et al., 2005).

3 Major Importance of Boron and Calcium in Legume Symbiosis

The study of the interaction between B and Ca is an important topic of research on mineral nutrition of plants. The content of either nutrient influences the tissue distribution (Ramón et al., 1990) and the requirements of the other for optimal plant growth (Teasdale and Richards, 1990). Boron is required for plants

at micromolar concentrations, but it has been implicated in several physiological processes (Blevins and Lukaszewski, 1998; Brown et al., 2002): cell wall structure and synthesis; membrane structure and membrane associated reactions; reproduction; nitrogen fixation; and phenolics metabolism. The diversity of plant processes affected by B leads to pleiotropic effects on plant development due to a deficiency of this micronutrient. Although the primary role of B in plants is unknown, its special chemistry, with the capacity of borate anions to form stable covalent links with cis-diols of carbohydrate moieties of molecules, turning them functional, has been established as the basis of any function of B (Bolaños et al., 2004b).

Calcium is also implicated in a large number of physiological processes in plants (Leonard and Hepler, 1990). Although the traditional functions of calcium in plants are also related to cell wall structure and membrane structure and function, recent reviews have focused on cytosolic free Ca^{2+} as one of the most important messengers involved in signal-response coupling (Rudd and Franklin-Tong, 2001; Sanders et al., 2002). Although most of plant Ca is linked to the cell wall and membrane, maintaining a small amount of cytosolic free Ca^{2+} is important because several physiological processes are accompanied by changes in cytoplasmic calcium concentration (Trewavas and Malhó, 1998). Moreover, a number of external stimuli lead to changes in cytosolic Ca^{2+}, which acts as a second messenger in the signaling between environmental factors and plant responses (Bush, 1995). Our previous studies in cyanobacteria demonstrated that calcium is implicated in the stability of heterocyst envelopes, and consequently in the protection of nitrogenase activity under stress conditions (Fernández-Piñas et al., 1995). Moreover, calcium may be involved in early signalling in reponse to temperature shocks, salinity, and osmotic stress in cyanobacteria (Torrecilla et al., 2000, 2001); and in heterocyst differentiation (Torrecilla et al., 2004). Therefore, a major challenge for future research will be to identify cellular targets of Ca^{2+} signals for cell differentiation and the primary sensors that perceive stresses and trigger Ca^{2+} signalling.

Evidence of a physiological B–Ca interaction was described for transport processes across the cell membrane (Tang and De la Fuente, 1986), although most of the investigations regarding the B–Ca relationship have been focused on the structure and function of the cell wall. In that sense, Kobayashi et al. (1999) demonstrated that Ca^{2+} promoted in vitro formation of dimers of borate-rhamnogalacturan II, and proposed that Ca^{2+} stabilizes pectin polysaccharides of the cell wall through ionic and coordinate bonding in the polygalacturonic acid region. At the signal transduction level, B could be implicated in the liberation of cytosolic Ca^{2+} by the cyclic-ADP ribose pathway (as proposed by Eckhert et al., 2007, following studies on prostate cancer) that regulates, among other things, ABA signaling (Wu et al., 1997).

Concerning nitrogen fixing organisms, a nutritional relationship between B and Ca in cyanobacteria was shown by our group. These microorganisms require B for maintaining the envelope of specialized N_2-fixing heterocysts (Bonilla et al., 1990). A supplement of extra Ca to cultures growing in the absence of B (Bolaños et al., 1993), or the addition of B to Ca-deficient treatments (Bonilla et al., 1995) resulted in a recovery of heterocyst structure and nitrogenase activity. In rhizobia-legume

symbiosis the studies of Carpena et al. (2000) suggest a specific B–Ca relationship in nodulated pea plants; they described the effect of low B on Ca concentration and a Ca-mediated mobilization of B from old to new growing tissues in B-deficient plants.

3.1 Boron and Nitrogen Fixing Rhizobia-Legume Symbioses

Legume (and also actinorhizal) N_2-fixing symbioses involve the development of a new plant organ, generally in the root, the nodule. New synthesis and deposition of wall and membrane material occurs to build a nodule; and several rhizobia and plant macromolecules decorated with cis-diol rich glycosil-moieties are implicated in plant-bacterial cell surface interactions. Therefore, B is a clue element in the establishment and maintenance of these symbioses.

The requirement of B for symbiotic N_2 fixation in legumes was very early suggested by Brenchley and Thornton (1925) in *Vicia faba* and confirmed at the end of the past century in nodulated *Pisum sativum* (Bolaños et al., 1994) (Fig. 1) and *Phaseolus vulgaris* (Bonilla et al., 1997a) plants (Fig. 2). In nodulated legumes, B deficiency led to a high reduction of nodules and nitrogenase activity (Table 1). Besides the extensive synthesis of new membranes and walls, rearrangements and changes in cell wall structure during nodule development explain a high requirement for B and typical symptoms of B-deficiency in the structure of nodules developed without B (Brewin, 2004). Studies at a molecular level have revealed that hydroxyproline-/proline-rich glycoproteins (Bonilla et al., 1997a) and pectin polysaccharides (Bonilla et al., 1997b; Redondo-Nieto et al., 2003) are abnormally assembled, leading to aberrant nodule cell walls.

Not only cell wall components, but also bacteria and plant-derived glycoconjugates containing cis-diol groups able to interact with borate anions, play essential roles in the correct establishment of the symbiosis between legumes and rhizobia

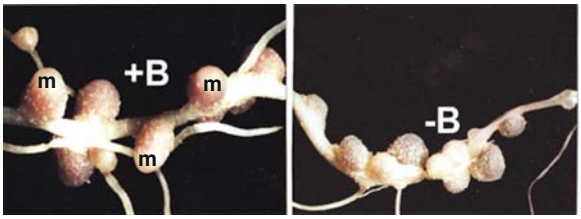

Fig. 1 Effects of B deficiency on pea (*Pisum sativum* L.) nodule development. Pea plants were grown with (+B) or without (−B) boron and inoculated with *Rhizobium leguminosarum* bv. *Viciae*. Note that 3 weeks post inoculation, nodules from B-sufficient plants have a colourless apical meristem (m) and a central zone of a red color due to the presence of the oxygen carrier leghemoglobin. By contrast, nodules from B-deficient plants are of a small size and of a pale color, because they develop abnormally in the absence of B and do not have leghemoglobin, being not functional. +B plants were fed with a growth solution containing 9.3 μ M B; −B plants were grown with no added B

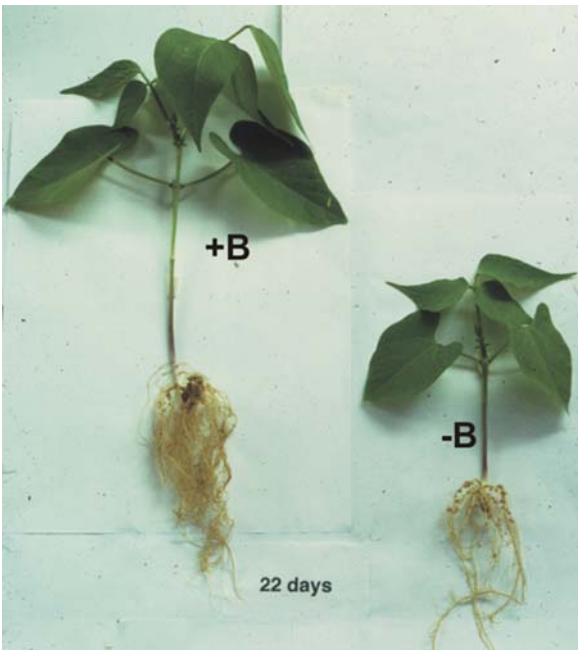

Fig. 2 Effects of B deficiency on the development of bean (*Phaseolus vulgaris* L.) nodulated plants 22 days after inoculation with *Rhizobium etli*. +B with boron, −B: without boron. B-deficiency led to poor nodulation and root development. +B: plants were fed with a growth solution containing 9.3 μ M B; −B: plants were grown with no added B

(Kannenberg and Brewin, 1994). Therefore, a B function is expected not only to stabilize nodule structure but also to modulate bacteria-plant interactions for the maintenance of a correct symbiotic relationship.

A legume-rhizobia symbiosis starts with the exchange of diffusible signal molecules between both partners, resulting in the activation of rhizobial *nod* (nodulation) genes in response to flavonoids exudated by the legume root (Spaink, 2000). The products of *nod* gene activity are the Nod (nodulation) factors, lipochitin-oligosaccharides that induce root hair deformation, cortical cell division (Dénarié and Cullimore, 1993), and preinfection structures in curled root hairs (van Brussel

Table 1 Effects of B deficiency (–B) on nodulation and nitrogenase activity of several legumes 4 weeks post inoculation with the appropriate host rhizobial strain. Nitrogenase was measured as acetylene reduction activity (ARA)(100% corresponds to 183 ± 36, 621 ± 137, and 8.1 ± 3.6 nmol C_2H_2 $root^{-1}h^{-1}$ for *Pisum sativum, Phaseolus vulgaris,* and *Medicago sativa,* respectively). The normal B treatment (+B) was 9.3 μM added as boric acid (H_3BO_3)

	Nodules per root		% Nitrogenase (ARA activity)	
	+B	−B	+B	−B
Pisum sativum-Rhizobium leguminosarum	170 ± 37	53 ± 26	100%	15%
Phaseolus vulgaris-Rhizobium etli	328 ± 65	143 ± 43	100%	28%
Medicago sativa-Sinorhizobium meliloti	17 ± 6	5 ± 3	100%	0%

et al., 1992) in the appropriate host legume. Boron deficiency leads to a very much reduced nodulation because of the very low *nod* gene induction activity of root exudates from B-deficient legumes (Redondo-Nieto et al., 2001). This effect might be a reflection of the phenolic, and hence flavonoid, metabolism, which is affected by boron nutrition (Ruiz et al., 1998), so that B deficiency can modify the presence or release of flavonoid compounds that in turn induce the expression of *nod* genes. Besides diffusible signals, colonization of the root surface by rhizobial cells is also diminished in B-deficient plants (Redondo-Nieto et al., 2001).

Following early preinfection plant-rhizobia signalling, induction of cortical cell division by Nod factors leads to a nodule primordium. Meanwhile, rhizobia colonizing curled root hairs make contact with the plant cell surface and invade the plant through a transcellular tunnel (the infection thread) sheathed with cell wall material (Rae et al., 1992), followed by an endocytosis-like process from unwalled infection droplets (Brewin, 1991, 2004). Infection threads in B-deficient legumes are extremely enlarged and aborted prior to bacterial release (Bolaños et al., 1996), even in the root hair that has previously reached the cortical cell (Redondo-Nieto et al., 2001). Furthermore, both indeterminate (pea) and determinate (bean) nodules appear almost uninvaded when they are induced in the absence of B (Bolaños et al., 1994; Bonilla et al., 1997a). During the growth of infection threads, rhizobia are embedded by a matrix containing plant-derived glycoproteins, including a root nodule extensin-like glycoprotein apparently important for intra- or intermolecular cross-linking (Rathbun et al., 2002). Boron can modulate interactions between the infection thread matrix glycoproteins and the bacteria cell surface, to promote nodule invasion (Bolaños et al., 1996). In the absence of B, root nodule extensin can attach to the cell surface of rhizobia. Therefore, the bacterium can be trapped, avoiding the endocytosis process leading to poorly invaded nodules.

After gaining the cytosol compartment, rhizobia are now called bacteroids, and are surrounded by a plant-derived membrane (peribacteroid membrane). They grow, divide, and develop into differentiated symbiosomes when biological nitrogen fixation takes place. The peribacteroid membrane harbors a differentiated glycocalyx composed of glycoproteins and glycolipids which codifferentiate with bacteroids (Perotto et al., 1991), and several thousand symbiosomes occupy each infected cell; consequently, an extensive synthesis and differentiation of membrane takes place at rates about 30- to 50-fold higher than in other tissues (Robertson and Lyttleton, 1984). During symbiosome maturation, new proteins are targeted to the symbiosome compartment to constitute a peribacteroid fluid. Two isoforms of a nodule-specific lectin-like glycoprotein (*Pisum sativum* nodule lectin, PsNLEC-1) seem to be implicated in bacteroid maturation (Dahiya et al., 1997; Sherrier et al., 1997). It has been shown that the glycosyl-moiety of these glycoproteins interacts with both the surface of the bacteroid and the symbiosomal membrane (Bolaños et al., 2004b). Therefore, this interaction seems to play a direct role for symbiosome development, since pea mutants lacking the symbiosomal form of PsNLEC 1 (Dahiya et al., 1998) or cell surface defective rhizobia (Perotto et al., 1994), that do not interact physically with PsNLEC 1, do not develop N_2-fixing bacteroids. The detection by specific antibodies of sugar groups of PsNLEC-1 demonstrates that the carbohydrate-moiety

of this protein was modified in the absence of B. Localization in ultra-thin pea nodule sections of PsNLEC-1 glycoproteins showed that they were accumulated in Golgi-derived or cytoplasmic vesicles, instead of symbiosomes in B-deficient nodules. This indicates a failure of the targeting of Ps-NLEC glycoproteins to the peribacteroid fluid of symbiosomes in B-deficient nodules (Bolaños et al., 2001). Aberrant bacteroid differentiation in the absence of B has been recently related to some glycoproteins that are possible borate ligands and that appear associated to the glycocalyx of the peribacteroid membrane of dividing symbiosomes. They are called RGII-glycoproteins, because they share antigenicity with rhamnogalacturonan II pectin polysaccharide (Redondo-Nieto et al., 2007). These glycoproteins were never detected in B-deficient cells, suggesting that they are stabilized on the glycocalyx through borate bridges and that association of the carbohydrate moiety of PsNLEC-1 with the peribacteroid membrane is mediated by RGII-glycoproteins.

Overall studies justify that boron is undoubtedly the micronutrient with the highest demand increase for symbiotic legumes. Although B is widely distributed both in the lithosphere and the hydrosphere, usually only soluble B (about 10% of total B in soil) is available to plants, making boron deficiency more common than deficiency in any other plant micronutrient worldwide (Shorrocks, 1997). Therefore, boron deficiency is a constraint for sustainable agriculture based on legume-rhizobia symbiosis; nevertheless, both the sufficiency and the toxicity of boron for nodulated legumes are in a narrow range of concentrations (Redondo-Nieto et al., 2003), and boron application following diagnosis of boron deficiency has to be extremely accurate.

3.2 Calcium and Nitrogen Fixing Rhizobia-Legume Symbioses

A role of Ca^{2+} for N_2-fixation in legumes was first reported by Greenwood and Hallsworth (1960). Later, Lowter and Loneragan (1968) described that a high Ca^{2+} supply was required to induce a high number of nodules in the plants, and Munns (1970) described a higher Ca^{2+} requirement for early infection events. These studies indicate an important role for Ca^{2+} in plant-bacteria signalling and recognition. The activity of *nod* genes is higher when the amount of Ca^{2+} for plant growth increases. Richardson et al. (1988) demonstrated that high Ca^{2+} increased the amount of *nod*-gene–inducing compounds in root exudates. This effect can be due to the role of Ca^{2+} on the synthesis of flavonoids. Application of external Ca^{2+} to plants enhances the phenylalanine ammonia-lyase activity (Castañeda and Pérez, 1996), the key enzyme in the flavonoid synthesis pathway.

Calcium is also required for an optimal root hair colonization (Lodeiro et al., 1995). Attachment of rhizobia is mediated by plant and bacterial components able to use Ca^{2+} as a ligand to reinforce the adhesion. Calcium ions can therefore strengthen the activity of plant lectins or rhizobial Ca^{2+}-dependent ricadhesines (Smit et al., 1989). Moreover, bacterial exopolysaccharide (EPS) can form a gel in the presence of cations as Ca^{2+}, being a non-specific mechanism for rhizobial attachment (Morris et al., 1989).

Besides early preinfection interactions, Ca^{2+} plays an important role in signal transduction during Nod factors perception and nodule organogenesis (Charron et al., 2004). Calcium has been demonstrated to act as a second messenger in Nod-factor signal transduction (Cárdenas et al., 2000; Lhuissier et al., 2001). Following Nod factor application, an influx of Ca^{2+} at the root hair tip (Felle et al., 1998) was the first detectable effect. This could lead to an efflux of Cl^- and membrane depolarization (Downie and Walker, 1999), causing an increase of cytosolic Ca^{2+} within a few minutes at the root hair tip (Cárdenas et al., 1999; Felle et al., 1999). First infection events, including root hair tip swelling, vacuolation, endoplasmic reticulum alignment with the plasma membrane, nuclear movement to the swelling, and inward growth of the cell wall to initiate the infection thread, can be related to these Ca^{2+} dynamics. About 3 min after Nod factor application, a reorganization of the actin cytoskeleton starts (Cárdenas et al., 1998). The cascade involved in the transduction of Nod factor signalling is mediated by a G-protein and phospholipases C (Pingret et al., 1998) that are also fully activated by Ca^{2+}. Hydrolysis of phosphatidylinositol biphosphate (PIP_2) by phospholipases C produces water soluble IP_3 that can regulate actin binding proteins (ABP)-mediated rearrangements of actin filaments and bundles. Furthermore, there are other later Ca^{2+} spikes originating from the perinuclear region of the root tip approximately 9 min after Nod factor application, which extend for at least 60 min–3 h (Ehrhardt et al., 1996). Although the role of these spikes is still unclear, there is some information concerning gene expression (Schultze and Kondorosi, 1998; Felle et al., 1999) that is important for cell cycle regulation during nodule organogenesis.

3.3 B–Ca Relationship in Biological Nitrogen Fixation

As stated above, investigating the roles of boron in nitrogen fixation, we demonstrated a relationship between the micronutrient and calcium in cyanobacteria, but also in nodulated legumes. Rhizobia-legume symbiosis is highly influenced by B and Ca^{2+} at the different steps of nodule development and organogenesis. Particularly important is the recovery effect of B deficiency by addition of Ca^{2+}, which is translated to a plant, mainly root, development (Fig. 3). Pea, bean, and alfalfa plants grown in media containing different concentrations of B and Ca^{2+}, and inoculated with their host rhizobia, develop different amounts of nodules and nitrogen-fixing activity, depending of the level of either nutrient in the growth media (Table 2), indicating that the relationship between B and Ca can be clearly stated (Redondo-Nieto et al., 2003).

Determination of *nod* gene activity, which is very low after exposure of *Rhizobium* to root exudates derived from B-deficient plants, demonstrates a higher induction capacity in plants treated with high concentrations of Ca^{2+}. Besides the exchange of diffusible signals, Ca^{2+} can also increase root colonization by rhizobia, which is diminished by B deficiency. Moreover, the phenomena of cell invasion and spreading inhibited by B deficiency were also recovered by addition of Ca^{2+}. However, Ca^{2+} could not prevent alterations of B-deficient nodule cell wall structure,

Fig. 3 Effects of different B and Ca treatments on the development of pea (*Pisum sativum* L.) (*upper side*) and bean (*Phaseolus vulgaris* L.)(*bottom side*) nodulated plants. Note that deficiency of B (−B+Ca treatment) led to poor nodulation and root development, and that supplementation with Ca (−B+2Ca treatment) partially prevented the effects of B deficiency and resulted in increased nodulation and root development. +B: 9.3 μ M B; +Ca: 0.68 mM Ca^{2+}

confirming that both nutrients are essential for wall architecture Ca^{2+} (Redondo-Nieto et al., 2003). Furthermore, previous results indicate that Ca^{2+} cannot prevent abnormal PsNLEC-1 targeting to the symbiosomal compartment under B deficiency (Redondo-Nieto, 2002), indicating a specific role for B in Golgi-derived vesicle targeting. Fig. 4 summarizes all of these results.

Table 2 Effects of different B and Ca treatments on nodulation and nitrogenase activity of nodulated *Pisum sativum* L. plants. Nitrogenase was measured as acetylene reduction activity (ARA)(100% corresponds to 183 ± 36 nmol C_2H_2 $root^{-1}h^{-1}$). The normal B treatment (+B) was 9.3 μM and the toxic treatment ++B was 46.5 μM, added as boric acid (H_3BO_3). The normal Ca treatment (+Ca) was 0.68 mM added as calcium chloride ($CaCl_2$)

	Nodules per root			% Nitrogenase activity (ARA)		
	+B	−B	++B	+B	−B	++B
+Ca	170 ± 37	53 ± 26	77 ± 31	100%	15%	26%
−Ca	22 ± 11	26 ± 10	18 ± 12	20%	10%	43%
+2Ca	267 ± 51	245 ± 64	231 ± 56	80%	50%	21%

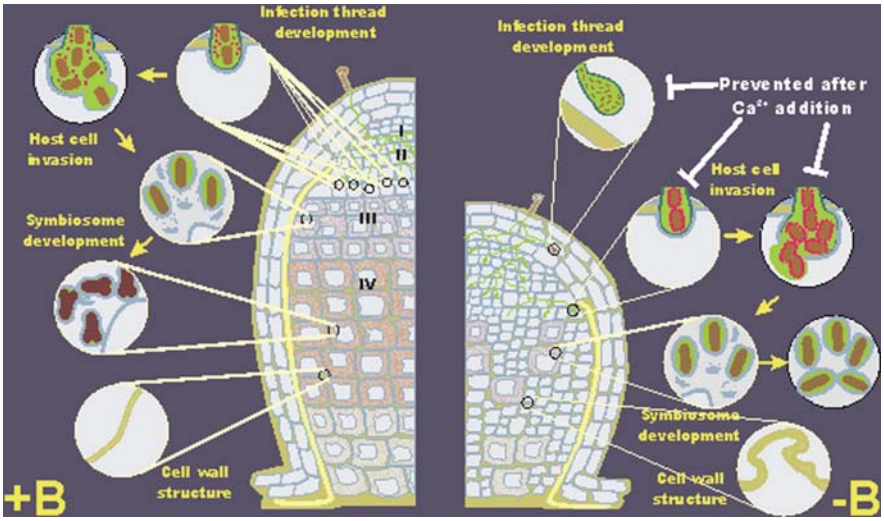

Fig. 4 Effects of B deficiency on the different processes of nodule development in the legume-rhizobia symbiosis (*left panel*). Prevention of B deficiency by Ca^{2+} addition (*right panel*). Boron deficiency inhibits infection threads development, reducing nodule invasion. Both effects are prevented by increasing Ca^{2+} supply. Therefore, Ca^{2+} appears more important than B during early events of nodule development. The absence of B also alters symbiosome differentiation and nodule organogenesis, leading to non−N_2-fixing nodules. Different zones of nodule development (I: nodule meristem, II: infection thread development, III: bacteroid proliferation, and IV: nitrogen fixation) are not clearly differentiated in B deficient nodules. Therefore, nodule organogenesis and maturation are absolutely B-dependent because deficiency effects on these processes cannot be prevented by Ca^{2+}. Finally, both nutrients are essential for maintaining nodule cell wall structure

Another insight into a B–Ca relationship comes from the study of gene expression during nodule development. Genetic studies of nodulation of *Medicago truncatula* showed that expression of more than 60% of the analysed genes (including genes involved in the cell cycle, cell wall assembly, and ribosome biogenesis) was affected by boron deficiency; and that, in some cases, a supplement of Ca^{2+} could reverse gene expression to a normal level (Redondo-Nieto et al., 2002). The

cyclic-ADP ribose pathway involved in release of internal Ca^{2+} is presumably modulated by B (Eckhert et al., 2007). Investigating whether those genes regulated by a B–Ca ratio are influenced by the cyclic-ADP ribose pathway will shed new light on the role of B in signal transduction.

Cyclic-ADP ribose is the central mediator of plant signaling involving abscisic acid (ABA), the primary phytohormone that mediates plant responses to stresses such as cold, drought, and salinity signaling (Wu et al., 1997). Interestingly, the interaction between calcium and boron seems to be more evident and important in nodule development and nitrogen fixation under salt stress. While pea plants cultivated under saline conditions did not develop nodules in a normal nutrient solution, modifying levels of B and Ca could increase nodulation and nitrogen fixation and recover plant development to 70% of that of plants grown without salt stress (El-Hamdaoui et al., 2003).

4 Conclusion

The knowledge of nutritional requirements and the role of different mineral nutrients during each step of the development of a legume-rhizobia symbiosis, as well as the impact of mineral nutrients on the process of nitrogen fixation, are imperative in a context of sustainable agriculture. Depending on the soil features, a particularly important factor for optimization of symbiotic N_2-fixation is the availability of phosphorus, potassium, iron, and molybdenum. Infection with vesicular-arbuscular mycorhizas improves phosphorus uptake; therefore, the "triple symbiosis" legume-rhizobia-mycorhiza will reduce N- and P-chemical fertilizer. Nevertheless, sustainable agriculture has to include a few of the conventional farming practices in a controlled way to optimize crop production without ecological risk. That is the case with potassium fertilization in saline soils; or the application of micronutrients with reduced availability due to the nodule environment or to the nitrogen fixation process, like iron or molybdenum; or those with a higher requirement for the development of symbiosis or the nitrogenase function, like cobalt or nickel. Our studies have demonstrated during the last two decades that boron is certainly the micronutrient whose deficiency has the most impact in nodule development and nitrogen fixation in legume symbioses. Because boron deficiency is very common worldwide, the diagnosis of B availability is very important prior cultivation of nodulated legumes. Our studies also show a relationship between boron and calcium during legume nodulation and symbiotic nitrogen fixation, both under physiological and under stress conditions. Nodulation and nitrogen fixation in legume-*Rhizobium* symbioses is dependent on B and Ca^{2+}. During the early events of nodulation, B was essential for *nod* gene induction, root hair curling, and adsorption of bacteria to the root surface, though Ca^{2+} addition could prevent the inhibitory effects of B deficiency and increased nodule number. High concentrations of Ca^{2+} also enhanced cell and tissue invasion by *Rhizobium*, which were highly impaired by B deficiency, although Ca^{2+} could not restore nodule structure. Taking into account that boron concentrations leading to either sufficiency or toxicity are quite precise, small calcium supplements

can be used for the correction of boron deficiency without a high application of B-fertilizer, mainly at the early stages of nodule development when Ca^{2+} can partially prevent B-deficiency.

Furthermore, the study of symbiosis under salt stress indicates that proper B and Ca nutrition can facilitate salt tolerance. Therefore, such studies should accompany genetic approaches searching for tolerant cultivars, in order to establish the best nutritional conditions for each type of legume, which will ensure the success of symbiosis, plant development, and crop production in saline soils.

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Uncommon Heavy Metals, Metalloids and Their Plant Toxicity: A Review

Petr Babula, Vojtech Adam, Radka Opatrilova, Josef Zehnalek, Ladislav Havel and Rene Kizek

Abstract Heavy metals represent a group of dangerous pollutants, to which is paid close attention. Many heavy metals are essential as important constituents of pigments and enzymes—mainly zinc, nickel, and copper. However, all metals, especially cadmium, lead, mercury, and copper, are toxic in high concentrations because they disrupt enzyme functions, replacing essential metals in pigments or producing reactive oxygen species. The toxicity of less common heavy metals and metalloids, such as thallium, arsenic, chromium, antimony, selenium, and bismuth has been investigated. Here we review the phytotoxicity of thallium, chromium, antimony, selenium, bismuth; and other rare heavy metals and metalloids such as tellurium, germanium, gallium, scandium, gold, platinum group metals (palladium, platinum, and rhodium), technetium, tungsten, uranium, thorium; and rare earth elements such as yttrium, lanthanum, and 14 lanthanides—cerium, dysprosium, erbium, europium, gadolinium, holmium, lutetium, neodymium, promethium, praseodymium, samarium, terbium, thulium, and ytterbium.

Keywords Heavy metals · Plant · Phytoremediation

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1 Introduction

The fate of heavy metals in the environment, as well as their toxicity and other properties, are still topical. This fact is well-documented judging from the number of articles where the "plants and heavy metal" term has been found within titles, abstracts, and keywords (Fig. 1). The interest is probably related to concerns about ensuring sufficient foodstuffs. Moreover, there have been developing technologies that can remediate an environment polluted by heavy metals. The technologies that use plants for this purpose are called phytormediation technologies (Macek et al., 2008). The plants are affected by many factors (physical, chemical, and biological). The simplified scheme of interactions between a plant and its environment is shown in Fig. 2. One of the groups of compounds affecting plants are heavy metals (Fig. 3). A heavy metal is a member of an ill-defined subset of elements that exhibit metallic properties, which would mainly include the transition

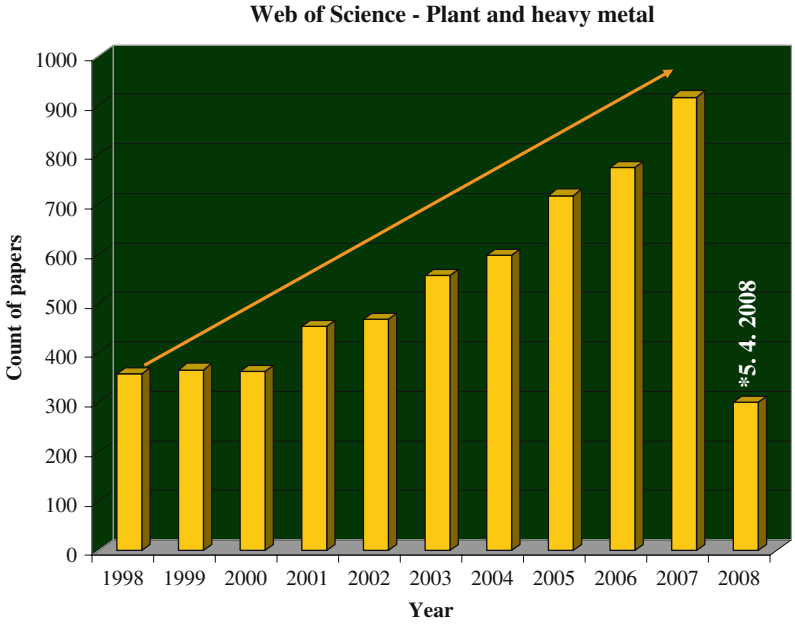

Fig. 1 The count of the published papers (5. 4. 2008, according to Web of Science) where the term "Plant and heavy metal" has been found within article titles, abstracts, and keywords

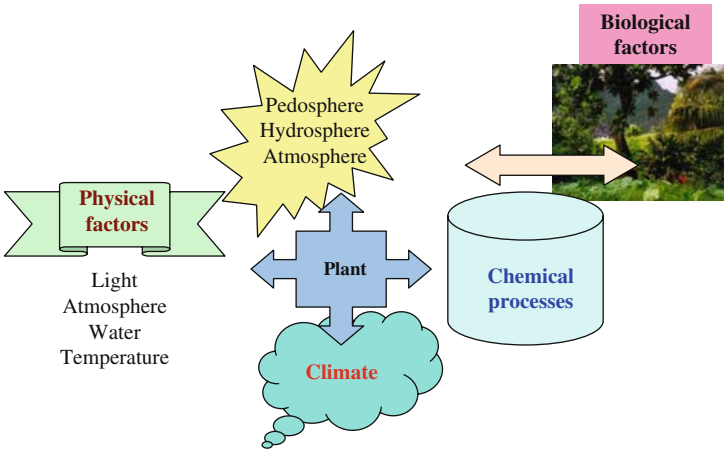

Fig. 2 The affecting of a plant by physical, chemical, and biological factors

metals, some metalloids, lanthanides, and actinides. They are widely distributed in the Earth's crust. Heavy metals may be retrieved from rocks of igneous (of volcanic origin), sedimentary (formed in layers by sedimentation), or metamorphic (transformed by intense heat and pressure) origin that contain specific elements. Heavy metals weathered from natural rock formations are widely spread in

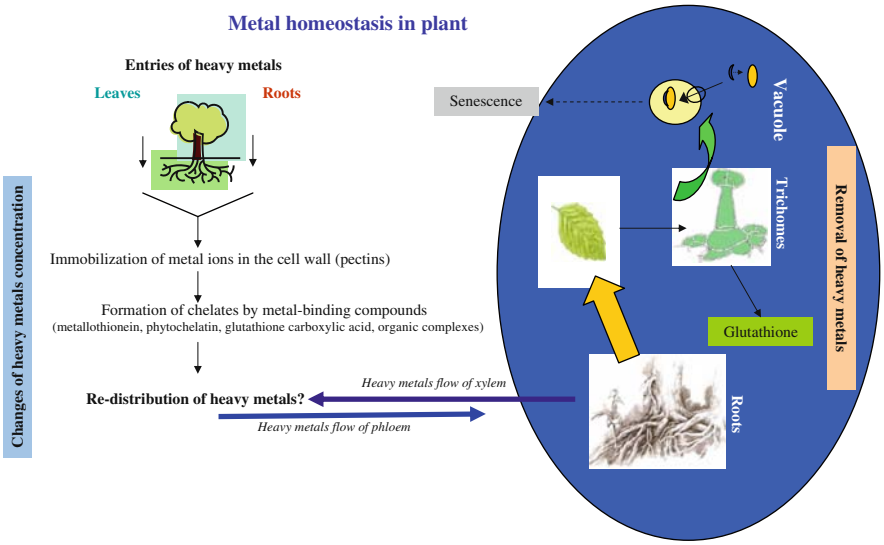

Fig. 3 The simplified scheme of influence and fate of heavy metals in a plant

the environment, occurring in particulate or dissolved forms in soils, rivers, lakes, seawater, and sea-floor sediments. Volcanoes also release heavy metals into the atmosphere. However, in areas of agricultural and industrial activity, higher concentrations of heavy metals (in comparison with background levels) can be detected. Soils near heavy-metal mines are especially exposed to the stress related to heavy metal, as well as metalloid pollution by Zn, Pb, Cr, Mn, Fe, Tl, In or As (Cabala and Teper, 2007). Chemical forms of heavy metals are still investigated to evaluate their possible mobility, bioavailability, and toxicity in living environments. Mainly reducible fractions of heavy metals and metalloids constitute potential risks to living, especially because of their solubility in aquatic environments (Boughriet et al., 2007).

Although many heavy metals are essential constituents of pigments and enzymes (mainly Cu, Ni, Zn, Co, Fe and Mo) for algae and plants, all metals/metalloids – but especially cadmium (Cd), lead (Pb), mercury (Hg), and copper (Cu) – are toxic in higher concentrations because they disrupt enzyme functions, replace essential metals in pigments, or produce reactive oxygen species. The similarity of certain heavy metals to essential heavy metals (for example, couples Cd−Zn, Se−S, or As−P) predestinates their high toxicity due to the possibility of replacing essential metals in enzymatic systems. The toxicity of less-common heavy metals and metalloids, such as thallium (Tl), arsenic (As), chromium (Cr), antimony (Sb), selenium (Se), and bismuth (Bi), is still being investigated.

2 Heavy Metal and Metalloid Uptake by Plants and Their Bioavailability

The important factor in the bioavailability of metals/metalloids is their presence in soil and water; there are not many plants that are able to uptake metals from the air. The next very important factor is the actual form of heavy metal (valence) in soil or water that matches the actual conditions, such as pH, oxygen content, and the presence or absence of other inorganic or organic compounds. There is no systemic correlation between soil metal content and the content of this metal in plant tissues. Some heavy metals are almost absolutely unavailable for plants due to their unsolubility and interaction with soil particles. The most suitable example is lead (Pb), which is present in big amounts in exposed areas but almost unavailable to plants because of its low solubility and strong interactions with soil particles (Nriagu and Pacyna, 1988). The ability of metals and metaloids to form complexes with compounds presented in water and soil that increase their bioavailability and uptake plays an important role. Heavy metals and metalloids can enter plants via up-take systems for essential cations, including different metal transporters (Eide, 2004; Guerinot, 2000; Perfus-Barbeoch et al., 2002; Shenker et al., 2001). Another very important role is that heavy metals and metalloid ions up-take is enabled by low molecular-weight compounds that are actively secreted by the roots of plants and serve as chelators (Shenker et al., 2001).

3 Transport of Heavy Metals and Metalloids in Plants

Heavy metals, as well as metalloids, are often accumulated in some plant organ/organs but not other plant organs; this fact is often species specific (McLaughlin et al., 1999; Wagner, 1993). A still-unanswered question is how heavy metals are transported to the xylem part of vascular bundles by radial transport involving radial passage across rhizodermis and endodermis with Casparian strips and their "efflux" from xylem parenchyma cells that provide transport for short distances to xylem via conductive elements (tracheids and vessels) followed by vertical transport to the aerial parts; it means to the vegetative as well as generative plant organs (Clemens et al., 2002). Some studies have proven that many heavy metals/metalloids are transported bound to low as well as high molecular-weight ligands, especially sulphur ligands (e.g., glutathione and phytochelatines—proteins derived from glutathione) and perhaps organic acids (Grill et al., 1989, 1985; Lugon-Moulin et al., 2004) as shown in Fig. 4. Low molecular-weight complexes of heavy metals/metalloids can be stored in vacuoles of root parenchymatic cells where they are transported with the help of specific transporters (Ortiz et al., 1992; Salt and Rauser, 1995; Salt and Wagner, 1993). But how are heavy metals/metalloids transported via xylem? Some works demonstrate that they are bound to oxygen or nitrogene ligands; metal and metalloid ions are transported across the cytosol of parenchyma cells into vascular cells due to the activity of p-type ATPases (Axelsen and Palmgren, 2001; Salt et al., 1995). The efflux of heavy metal ions/metalloids into cells of target tissues plays an important role with similar mechanisms.

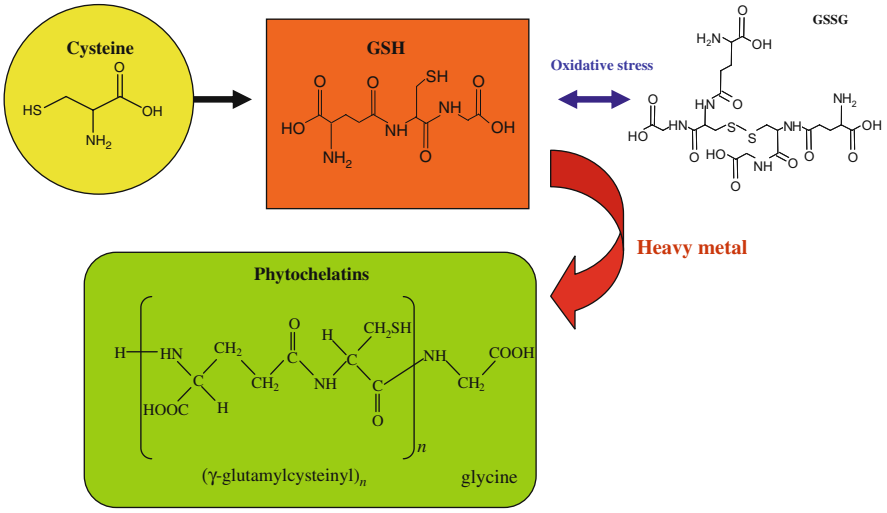

Fig. 4 The chemical structure of cysteine, reduced glutathione (GSH), oxidized glutathione (GSSG), and phytochelatins (PCs). The GSH/GSSG ratio closely relates to oxidative stress connected with the presence of reactive oxygen species. Besides, synthesis of phytochelatins is a plant-cell response to the presence of heavy metal ions within a cell

4 Metals/Metalloids Toxicity and Tolerance

The symptoms of metal/metalloid toxicity are similar, and the most investigated heavy metal is cadmium. The most important effects of heavy metals/metalloids are:

- Oxidative stress, because of the oxidative-redox properties of many heavy metals and metalloids (DeVos et al., 1992; Supalkova et al., 2007)
- Bond of heavy metals/metalloids to the structures of proteins and other bioactive compouds due to their similarity to essential metals

Plant response to the presence of heavy metal/metalloid ions includes the synthesis of plant thiol compounds – namely phytochelatines (Adam et al., 2005; Klejdus et al., 2004; Petrlova et al., 2006; Potesil et al., 2005; Rauser, 1995; Supalkova et al., 2008, 2007; Vatamaniuk et al., 2000; Zehnalek et al., 2004a, b; Zitka et al., 2007). Some plants, called metallophytes, demonstrate tolerance or hypertolerance to heavy metals/metalloids, as well as hyper-accumulation of one or more metals/metalloids. These plants may have two important economic possibilities – phytomining (heavy metal extraction) and phytoremediation (metal accumulation from soil in plants). There are several processes of phytoremediation:

- Phytoextraction – accumulation of heavy metals from soils in plan organs that can be harvested
- Rhizofiltration – decontamination of polluted waters and seawage by adsorbing or up-taking the roots of plants
- Phytodegradation – utilization of the ability of some plants to decompose (degrade) pollutants
- Phytostabilization – storage of heavy metals or other pollutants in plant tissues in the form of complexes with limited solubility
- Phytovolatilization – detoxification of soils by plants with the ability to produce volatile compounds

Definite mechanisms of hypertolerance are still unknown, but some genes – especially for metal homeostasis and stress genes – were identified as responsible (Weber et al., 2004). Phytoremediation technologies can be used to treat environmental problems. One of the main advantages is that the cost of phytoremediation is lower than that of traditional processes, both in situ and ex situ. In the case of remediation of environments polluted by organic compounds, they can be degraded. Moreover, there is also the possibility of the recovery and re-use of valuable metals (Fig. 5).

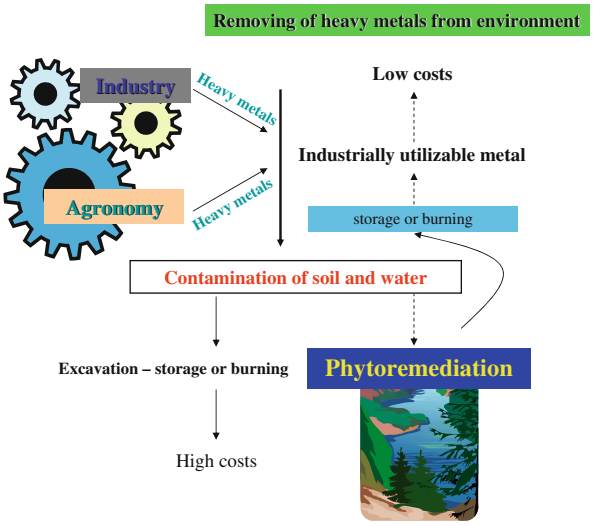

Fig. 5 The simplified scheme of the polluting of the environment by heavy metals with respect to different ways of remediation of the environment

4.1 Thallium

Thallium is a soft, bluish-grey, malleable heavy metalloid that was discovered in 1861 by Sir William Crookes. It is not a rare element; it is 10 times more abundant than silver. This metalloid occurs mainly in association with potassium minerals such as sylvite and pollucite in clays, soils, and granites. Thallium minerals are well-known too – they are rare, but a few are known, such as crookesite, lorandite, christite, avicennite, ellisite, or sicherite. They contain 16–60% thallium, namely as sulphides or selenides in complexes with antimony, arsenic, copper, lead, and silver (Anderson et al., 1999; Xiao et al., 2004).

Some thallium compounds, such as thallium sulphate, were used as rat poisons and insecticides in the 1970s. Some compounds are still used, especially in electronic equipment (solar cells, light sensitive crystals), infrared light detectors, and medical imaging devices (Kazantzis, 2000). This metalloid is also produced as a by-product of coal mining, the zinc and nonferrous industry (lead smelting), and in cement factories (Tremel and Mench, 1997). Thallium is partially water-soluble and consequentially can spread with groundwater when soils contain large amounts of the component. Thallium can also spread by adsorption on sludge. There are indications that thallium is fairly mobile within soils. When it enters the environment, it doesn't break down and is absorbed by plants. It then enters the food chain and can accumulate in fish and other animals and demonstrate its toxicity (Al-Najar et al., 2003; Kwan and Smith, 1991; Lin et al., 2005).

Thallium is not an ubiquitous element and is itself very toxic – its salts are considered to be the most toxic compounds known. The most important valence state

Mineral	Chemical formula	Mineral	Chemical formula
Avicennite	Tl_2O_3	Lorandite	$TlAsS_2$
Bernardite	$TlAs_5S_8$	Parapierrotite	$Tl_2(Sb, As)_{10}S_{16}$
Carlinite	Tl_2S	Picotpaulite	$TlFe_2S_3$
Chabourneite	$Tl_{21-x}Pb_{2x}(Sb, As)_{91-x}S_{147}$	Pierrotite	$Tl_2(Sb, As)_{10}S_{16}$
Christite	$TlHgAsS_3$	Raguinite	$TlFeS_2$
Chalcothallite	$(Cu, Fe, Ag)_6.3(Tl, K)_2SbS4$	Rathite	$(Pb, Tl)_3As_5S_{10}$
Crookesite	Cu_7TlSe_4	Rebulite	$Tl_5Sb_5As_8S_{22}$
Edenharterite	$TlPbAs_3S_6$	Routhierite	$TlHgAsS_3$
Ellisite	Tl_3AsS_3	Sicherite	$TlAg_2(As, Sb)_3S_6$
Fangite	Tl_3AsS_4	Simonite	$TlHgAs_3S_6$
Galkhaite	$(Cs, Tl)(Hg, Cu, Zn)_6(As, Sb)_4S_{12}$	Stalderite	$TlCu(Zn, Fe, Hg)_2As_2S_6$
Gillulyite	$Tl_2(As, Sb)_8S_{13}$	Thalcusite	$Cu_3FeTl_2S_4$
Hatchite	$AgPbTlAs_2S_5$	Thalfenisite	$Tl_6(Fe, Ni, Cu)_{25}S_{26}Cl$
Hutchinsonite	$(Pb, Tl)_2As_5S9$	Vaughanite	$TlHgSb_4S_7$
Imhofite	$Tl_6As_{15}S_{25}$	Vrbaite	$Hg_3Tl_4As_8Sb_2S_{20}$
Jankovicite	$Tl_5Sb_9(As, Sb)_4S_{22}$	Wallisite	$CuPbTlAs_2S_5$
Jentschite	$TlPbAs_2SbS_6$	Weissbergite	$TlSbS_2$
Lanmuchangite	$TlAl(SO_4)_2.12H_2O$		

of Tl is Tl(I). In this state, thallium forms many compounds with different solubilities that play crucial roles in bioavailability. The most soluble are thallium(I) thiocyanate, thallium(I) chloride, thallium(I) bromate, thallium(I) sulphate, thallium(I) acetate, thallium(I) carbonate, and thallium(I) bromide that are toxic. The least-soluble Tl compounds are thallium(I) sulfide and thallium(I) hydroxide, which are much less toxic in comparison with previous group (Moeschlin, 1980). Tl uptake by plants is almost entirely process-driven and is not connected with pH changes and ligand concentrations in plant cells. During transport, as well as in cytosol of plant cells, Tl (I) does not form complexes with other compounds and does not convert to other valence states such as Tl (III), which is typical for Tl organic compounds (Mestek et al., 2007). Inhibition of uptake was recorded in the presence of monovalent ions because of Tl similarity to alkali metals (Durrant and Durrant, 1970). Experiments carried out on *Lemna minor* demonstrate that Tl can be transported through the whole plant and can pass through plant cell walls and as well as plasmatic membranes. More than 80% of Tl is held in vacuoles (Kwan and Smith, 1991). The toxicity of thallium is probably based on its interactions with potassium, especially on its substitution in enzymatic systems such as (Na + /K+)-ATPase and other monovalent cation-activated enzymes, as well as a high affinity for sulfhydril groups of proteins and other biomolecules (Aoyama, 1989; Aoyama et al., 1988; Douglas et al., 1990). One work using mammalian cells as a biological model based on Tl interaction with sulhydrils groups of aminoacids L-cysteine and L-methionine demonstrated the enhancement of Tl toxicity in the presence of these aminoacids (so very important), but an unanswered question is what roles do plant thiol compounds play in the processes of thallium detoxication (Montes et al., 2007)?

High Tl content in soils because of high rock Tl content (geogenic origine) does not lead to the higher Tl content in plants due to its low bioavailability (Al-Najar et al., 2005), but Tl of anthropogenic origin represents a very available Tl form for plant up-take. The more intensive Tl transport was observed in the case of soil contamination with thallium(I) sulphate. Some investigations report that thallium can accumulate especially in plants of the *Brassicaceae* family (Al-Najar et al., 2005; Madejon et al., 2007). Some chemical analysis of two semiarid species (*Hirschfeldia incana* and *Diplotaxis catholica*) growing on Tl-contamined soils demonstrate Tl accumulation that was in correlation with precipitations. In dry years, Tl accumulation was significantly reduced compared to wet years (Madejon et al., 2007). Tl accumulation in plants is probably connected to soil type. Experiments with the other Tl hyper-accumulating species of the *Brassicaceae* family (kale – *Brassica oleracea subsp. acephala* cv. Winterbor Fl) and candytuft – *Iberis intermedia*) both showing unusual Tl hyperaccumulation (0.08% (w/w) Tl content in dry matter) – demonstrate the highest potential of Tl accumulation (Al-Najar et al., 2005, 2003; Kurz et al., 1999). These species can be usable for "phytomining" – growing a "crop" for recover of metals instead of subeconomic conventional mining (Leblans et al., 1999). Some works indicate that Tl transport into aerial plant parts is species specific. Some species (important crops) demonstrate Tl accumulation in stems (*Triticum ssp.*, *Zea mays*) and consequently low Tl accumulation in fruits; but some, such as *Brassica napus*, in contrast demonstrates high Tl accumulation in seeds and low Tl accumulation in stems and leaves (Tremel et al., 1997). Other species, and also important crops that are Tl accumulators, are rye grass (*Lolium perenne*, Poaceae–Graminae), rape (*Brassica napus* subsp. *oleifera*, Brasicaceae), and bush beans (*Phaseolus vulgaris*, Fabaceae–Leguminosae) (Makridis et al., 1996). Tl accumulation was also demonstrated in vegetables, such as carrot or celery (Kurz et al., 1999) that can create big problems because of the absence of threshold limits for thallium in soils, agricultural products, agricultural feed, and foodstuffs in most countries (Bunzl et al., 2001; Pavlickova et al., 2006).

Plant species/family	Tl accumulation
Brassica oleracea subsp. *acephala* cv. Winterbor/Brassicaceae	+++
Brassica napus subsp. *oleifera*/Brassicaceae	+++
Brasica napus/Brassicaceae	+++
Diplotaxis catholica/Brassicaceae	+++
Hirschfeldia incana/Brassicaceae	++
Iberis intermedia/Brasicaceae	+++++
Lolium perenne/Poaceae	++
Phaseolus vulgaris/Fabaceae	++
Triticum ssp./Poaceae	+/++
Zea mays/Poaceae	++

4.2 Chromium

Chromium is an essential microtrace element, or heavy metal, that is required for sugar metabolism in humans. In an elemental form, chromium occurs in nature very rarely, but plenty of minerals containing chromium are well-known; chromium is the seventh most abundant element on Earth (Katz and Salem, 1994). The only chromium ore with importance is chromite, which occurs in ulframafic and serpentine rocks; other minerals contain chromium in complexes with other elements, especially with lead, magnesium, or aluminium.

Mineral	Chemical formula	Mineral	Chemical formula
Bellite	$PbCrO_4$, AsO_4, SiO_2	Phoenicochroite	Pb_2OCrO_4
Bentorite	$Ca_6(Cr, Al)_2(SO_4)_3(OH)_{12} \cdot 26(H_2O)$	Stichtite	$Mg_6Cr_2CO_3(OH)16.4H_2O$
Chromite	$(Fe, Mg)Cr_2O_4$	Tarapacaite	K_2CrO4
Crocoite	$PbCrO_4$	Uvarovite	$Ca_3Cr_2(SiO_4)_3$
Knorringite	$Mg_3Cr_2(SiO_4)_3$	Vauquelinite	$CuPb_2CrO_4PO_4OH$
Lopezite	$K_2Cr_2O_7$	Zhanghengite	Cu, Zn, Fe, Al, Cr
Mariposite	$K(Al, Cr)_2(Al, Si)_4O_{10}(OH)_2$	Zincochromite	$ZnCr_2O_4$

Chromium is a common contaminant of surface waters and ground waters because of its occurrence in nature, as well as its utilization in the electroplating industry as an electroplating cleaning agent and in catalytic manufacturing, in refractories and drilling muds that produce a big amount of chromium salts. Chromium is highly soluble under oxidizing conditions and forms Cr(VI) anions, such as chromates CrO_4^{2-} or dichromates $Cr_2O_7^{2-}$. Under reducing conditions, Cr (VI) converts to Cr (III), which is insoluble, but this form is strongly absorbed onto the surface of soil particles. These two forms are most stable and common in terrestrial environments. The most important sources of Cr (III) are fugitive emissions from road dust and industrial cooling towers. Hexavalent chromium compounds are used in the manufacture of pigments, in metal finishing and chromium plating, in stainless steel production, in hide-tanning, as corrosion inhibitors, and in wood preservation (Shtiza et al., 2008). The solubility of chromium salts goes down in the range Cr (VI)− Cr (IV)− Cr (III).

Trivalent chromium is essential for animal and human health, whereas hexavalent chromium salts demonstrate high toxicity and strong carcinogenic effects and may lead to the death of exposed animals and humans. Chromium as chromate can be actively transported across biological membranes of prokaryotes by the mechanism of active sulphate transport that has been demonstrated on *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas fluorescens*, *Alcaligenes eutrophus*, and also on cyanobacteria *Anabaena doliolum* (Dreyfuss, 1964; Hryniewicz et al., 1990; Karbonowska et al., 1977; Pardee et al., 1966; Rai et al., 1992; Sirko et al., 1990).

Pollution of water environments by chromium salts presents an important problem for the industsry. Some algae demonstrate a chromium biosorption ability but

the data on chromium transport are very uncommon. The most important factor of biosorption was determined to be the pH of water, with a pH of 4.5 for Cr (III) and 2.0 for Cr (VI). These results imply the importance of the oxidative state of chromium for up-take (biosorption) (Murphy et al., 2008). It is obvious that the salinity of water as well as the presence of dissolved salts and concentration of Cr salts and their oxidative state markedly influence the ability of some microorganisms (namely *Micrococcus* sp. – that was isolated from waters highly contamined by chromium salts – *Scenedesmus, Pandorina, Cladophora, Cyanidium caldarium*, and cyanobacterium *Phormidium laminosum*) to bioaccumulate Cr (VI) salts (Kilic and Donmez, 2007; Sampedro et al., 1995; Vymazal, 1990). Many algae and aquatic microorganisms (genera *Spirogyra, Mougeotia, Chlorella, Scenedesmus, Selenastrum, Euglena*) demonstrate growth inhibition based on inhibition of respiration and photosynthesis, as well as cytoskeleton alterations in the dependence on Cr concentration and other effects evoked by Cr (III) and Cr (VI) salts (Brady et al., 1994; Brochiero et al., 1984; Fasulo et al., 1983; Liu et al., 1995; Travieso et al., 1999). The problem of Cr-bioaccumulation can be very important in the case of industrial-exploited algae species, namely *Gelidium* genus species because of their ability to bioccumulate chromium, especially as Cr (III) salts (Vilar et al., 2007). Accumulation of chromium was also determined in some aquatic and floating plants, such as *Eichhornia crassipes* (Mangabeira et al., 2004), *Hydrocotyle umbellata* (Yongpisanphop et al., 2005), or *Bacopa monnieri* (Shukla et al., 2007), that can be used for wastewater treatment. An important question is about the usage of wetland plants such as *Typha* sp., *Phragmites* sp., *Scirpus* sp. *Leersia* sp., *Juncus* sp., or *Spartina* sp., whose ability to reduce heavy metal levels in polluted waters is well-known (Baudo et al., 1985; Gupta et al., 1994).

Chromium can be absorbed as Cr^{3+} or CrO_4^{2-} by the roots of "higher" plants, but available data are still contradictory. Compared with the highly oxidized hexavalent form Cr (VI) (Cr-21), the Cr (III) form of plenty of compounds (especially hydroxides, oxides, or sulphates) is relatively less soluble and therefore less bioavailable but more stable (Srivastava et al., 1994). Contrary to this argument, the study of Huffman et al. demonstrated the no up-take differences between Cr (III) and Cr (VI) in beans *(Phaseolus vulgaris,* Fabaceae) and wheat (*Triticum aestivum*, Poaceae) (Huffman and Allaway, 1973). Reciprocal ratios of different Cr forms probably plays important role in Cr up-take. It was described that equal concentrations of Cr^{3+} and CrO_4^{2-} in a substrate lead to unavailability of both chromium forms for oak trees (McGrath, 1992). It seems that mycorhizzal fungi, as well as the ability of Cr salts to form more soluble complexes with organic acids, are able to increase chromium up-take by plants (Davies et al., 2002; Srivastava et al., 1999). Only very few studies have attempted to transport mechanisms and identify the chromium chemical forms in plants, but factors such as oxidative Cr state or its concentration in substrates play important roles (Kleiman and Cogliatti, 1998; Mishra et al., 1995). Chromium(VI) is probably transported by active transport thanks to sulphate carriers, but Cr (III) is transported passively by cation exchange sites of cell walls (Skeffington et al., 1976). Some studies indicate that plant supplementation by different chromium forms (trivalent, hexavalent) leads to the detection of

only hexavalent chromium in plant tissues (Skeffington et al., 1976), but some plants (such as soybean and garlic), as well as algae, have the capacity to reduce hexavalent chromium forms to intermediate Cr (V) and Cr (IV) forms that can be also detected, or eventually reduce to Cr (III). This represents a detoxication pathway of very toxic Cr (VI) forms, especially CrO_4^{2-}. Plant tissues (organs, shoots, roots) with known reduction capacities are still unknown (Hauschild, 1993; Katz and Salem, 1994; Liu et al., 1995; Micera and Dessi, 1988), but it is obvious that the specific reduction capacity to reduce CrO_4^{2-} ions to Cr^{3+} ions carries Fe(III)-reductase enzymes, which is validated by the fact that Cr application to -deficient plants increases the activity of root-associated Fe (III)-reductase (Schmidt, 1996). This conclusion confirmed the study of Zayed et al. that demonstrated the presence of only Cr^{3+} form in root tissues, and the absence of Cr (VI) in CrO_4^{2-} form after CrO_4^{2-} application – contrary to a previous study by (Skeffington et al., 1976; Zayed et al., 1998a). A study of other research confirmed the prevailing Cr^{3+} in Indian mustard (*Brassica juncea*) plant tissues after CrO_4^{2+} application (Bluskov et al., 2005; Dushenkov et al., 1995; Han et al., 2004).

Some workers reported that Cr (III) ions are highly stabilized by complex formation with organic molecules, such as proteins (glutathione), carbohydrates (especially pentoses), NAD(P)H, $FADH_2$, and probably also with organic acids, and stored in root cell vacuoles in precipitated form or in apoplast in cell walls, which is the reason for restricted mobility of chromium in plants (Mangabeira et al., 2004). Transport of chromium is probably restricted only to vascular tissues. A study utilizing tomato plants (*Lycopersicum esculentum*, Solanaceae) relieved its restriction to vascular tissues of roots (especially secondary xylem), stems, and leaves with localization inside in vessels, a very limited amount in xylem parenchymatic cells, and no transport to cortex or epidermis of stems or palisade/spongy parenchyma of leaves (Mangabeira et al., 2004). Chromium in vascular tissues is probably complexed with organic acids (Juneja and Prakash, 2005). An association of chromium ions with hydroxyl groups of cell walls is probable and can be the reason for no transport out of vascular tissues (Mangabeira et al., 2004). Studies carried out on important vegetable crops and other plants confirmed the ability of some of them (especially cauliflower, kale, and cabbage) to accumulate chromium (as CrO_4^{2-}, less as Cr^{3+}), mostly in their roots with general minimal chromium transport to aerial parts (Zayed et al., 1998a) because of their minimal entry to the vascular tissues (Zayed et al., 1998a). It is a very important determination that iron hyperaccumulators such as spinach appear to be the most effective Cr translocators to shoots compared to other plants (Cary et al., 1977).

A stimulative effect of chromium on plant growth in very low concentrations was demonstrated; its application to the soil increased the nitrogen fixation by some leguminosae plants and the growth ration of other plants (Hewitt, 1953). What are the mechanisms of Cr's toxic effects and their manifestations in plants? Chromium complexes can react with hydrogen peroxide and generate significant amounts of hydroxyl radicals that may directly trigger DNA alterations and other effects (Shi and Dalal, 1990a, b). The possibility of Cr (III) ions affect the processes of DNA replication and transcription are still being discussed (Bridgewater et al.,

1994; Costa, 1991; Kortenkamp et al., 1991; Nishio and Uyeki, 1985). Micronuclei formation and chromosome aberrations were observed in *Vicia faba* and *Allium cepa* exposed to heavy metals, including chromium (Minissi et al., 1998; Rank and Nielsen, 1998). Experiments carried out on model plants demonstrated a reduction of growth ratio (decrease of biomass production), chlorosis development (decrease of transpiration and photosynthesis rate demonstrated in *Nymphaea alba, Nelumbo lutea, Nymphaea spontanea*, *Spirodella polyrhiza*, etc.), and turning of stems with woody, higher content of proline in their leaves guided by proteosynthesis reduction and nitrate reductase decreases (Choo et al., 2006; Vajpayee et al., 1999a, b; Vajpayee et al., 2000; Vernay et al., 2007) and an increase in activity of some antioxidant enzymes, such as CAT, SOD, and POD, that can be connected with the ROS generation by Cr and play important role in protection (Karuppanapandian et al., 2006; Pandey et al., 2005). Chromium inhibits seed germination (Speranza et al., 2007) and expressively negatively influences the development of seedlings (Chanda and Parmar, 2003; Iqbal et al., 2001). Some of Cr's toxic effects resemble the Fe deficiency in plants (Agarwala et al., 1965; Wong and Chang, 1991). Chromium probably increases the availability of Fe for heme biosynthesis (Chereskin and Castelfranco, 1982; Pushnik and Miller, 1989). Microscopic analysis identified the thicker deposition of waxes on the leaves surface (Arduini et al., 2006) that is probably connected with the disruption of water transport from roots to other aerial plant parts. The relation between chromium application and secondary metabolite production (especially terpenes – essential oils) probably connects with growth processes reduction, as it was demonstrated in *Ocimum tenuiflorum* (Lamiaceae). Chromium application led to higher eugenol production (Rai et al., 2004).

Plants placed in the *Brassicaceae* family are known as "S-loving" plants (connection with ability of sulphur transport to the tissues and its metabolism?) and they generally represent Cr-hyperaccululators (Hsiao et al., 2007; Kumar et al., 1995) as well as plants growing on soils contamined by chromium salts such as *Herniaria hirsuta* (Shallari et al., 1998), *Sutera fodina, Dicoma niccolifera*, *Leptospermum scoparium*, *Genipa americana* (Rubiaceae; Barbosa et al., 2007), *Typha* spp. (Dong et al., 2007), *Amaranthus viridis* (Zou et al., 2006), miscanthus (Arduini et al., 2006), *Oryza sativa* (Bhattacharyya et al., 2005), *Convonvulus arvensis* (Gardea-Torresdey et al., 2004), *Leucaea leucocephalla* (Rout et al., 1999) and willows (*Salix* spp.; Yu and Gu, 2008). The addition of syntetic chelating agents (e.g,. EDTA) to substrates can increase the mobility and phytoavailability of Cr (Erenoglu et al., 2007; Yu and Gu, 2008). Mechanisms of Cr tolerance are still unknown, but they are probably connected with the ability to reduce Cr (VI) to Cr (III).

4.3 Antimony

Antimony is a metalloid that can exist in two different chemical forms – a metallic form and a nonmetallic form. Antimony occurs in the environment naturally, but it also enters the living environment thanks to human activities. Antimony occurs widely in nature; more than 100 minerals containing this metalloid are well-known.

Antimony itself is a very rare element, but it is far more common in sulphides and salts of sulphur. The predominate mineral is stibnite (Sb_2S_3), and other important minerals are aurostibite, kermesite, or valentinite. In environmental samples, antimony exists mainly as Sb (III) and Sb (V) (Filella et al., 2001).

Antimony emissions into living environments are exclusively due to human activities. The most important emitted antimony form is antimony trioxide, as the result of coal burning or ore smelting that contains antimony. The chemical behavior of antimony is very similar to arsenic because of its neighbor in the periodic table. Soluble antimony forms are quite mobile in the water; less soluble antimony species are adsorbed onto soil particles—they are mainly bound to iron and aluminium. The most important sources of antimony pollution in urban areas are abrasion of antimony from brakes, tires, and street surfaces, and the emission of antimony in vehicle exhaust (Merian, 1990; USEPA, 1979).

The toxicity of antimony is not well-known, but Sb(III) species are usually more toxic than Sb(V) species, and is comparable in its biochemical behavior with arsenic and bismuth. It seems probable that algae and plants with a high ability to accumulate As and Bi are also able to accumulate antimony. Some works are interested in the influence of antimony to microorganisms. An interesting species is *Chlorella vulgaris*, which demonstrates better growth parameters in medium supplemented with potassium tartrate in comparison with an antimony-poor medium. Its ability to bioaccumulate antimony is also interesting; 12 mg Sb to 1 g dry matter (Maeda et al., 1997). These results mean that toxic antimony(III) is converted to much less toxic antimony(V) in living cells, bound to low molecular-weight proteins and probably stored in vacuoles (Foster et al., 2005). Bioavailability of Sb is very low because of the very limited bioavailability of this element (Casado et al., 2007). There are no detailed studies interested in uptake, transport, or mechanisms of the toxic effect of antimony. We can suppose that mechanisms of antimony metabolism are similar to other heavy metals – after up-take, toxic Sb(III) form is converted to the Sb(V) less-toxic form, consequently complexed with proteins (phytochelatines?) or carbohydrates and stored in vacuoles of plant cells. In plant extracts from areas poluted by antimony from mining activities, organic, methylantimony compounds as well as inorganic forms were determined (Miravet et al., 2005). As potent Sb bioaccumulator *Dittrichia viscosa* (Murciego et al., 2007), *Digitalis purpurea, Erica umbellata, Calluna vulgaris* and *Cistus ladanifer* (Pratas et al., 2005), cyanobacteria and plants that bioaccumulate antimony from contamined waters due to their ability to grow partially submerged at least, such as *Ceratophyllum* ssp. (Hozhina et al., 2001) were determined as potent Sb bioaccumulators.

4.4 Selenium

Selenium is a nonmetallic chemical element that, in chemical behavior, resembles sulphur and tellurium. This metalloid appears in many allotropic forms. Selenium itself is very rare element on the Earth's surface. All 40 minerals containing

selenium are very rare and occur together with sulphides and metals such as copper, zinc, and lead. The most important selenium inorganic forms are selenides, selenates, selenites, and (rarely) elemental Se. Selenium occurs naturally in living environments and it is released due to natural processes (weathering of rocks) as well as human activities (Sharmasarkar and Vance, 2002). The most important selenium form that enters the air is selenium dioxide, originating in the processes of coal and oil combustion. This substance can be converted to other selenium forms, such as methyl derivates or selenium acid, and can be adsorbed on dust particles. Selenium from air as well as from waste (and fertilizers) tends to end up in the soil of disposal areas. Selenium behavior in soils and water is strongly dependent on its interactions with other compounds and environmental conditions. Selenium stays immobile in soil, but oxygen levels and acidity increase the amount of mobile selenium forms. Soil acidity increase is usually caused by human activities connected with industrial and agricultural processes; Se content in some commercially utilized fertilizers is controversial; only 5−30 % of Se applied like this is utilized by plants, the rest is retained in thhhe soil or a small part can be released into the atmosphere by the processes of volatilization (Mikkelsen et al., 1989; Tveitnes et al., 1996; Ylaranta, 1990).

Selenium is considered to be a trace element with fundamental functions in the antioxidant enzyme family of glutathione peroxidase (Rayman, 2000), but because of very small differences between Se essentiality and Se toxicity, it has the potential to accumulate at toxic levels in living environments – especially in aquatic ecosystems in algae and plants (Ibrahim and Spacie, 1990; Lemly, 2002) – and consequently can endanger the health of birds and fish (USEPA, 2004).

Selenium in soil represents an expressive problem – with changes in soil's environmental factors, it can become mobile. The most important factors of Se mobility in soils are pH, redox soil conditions, temperature, and the presence of inorganic as well as organic compounds (especially $CaCO_3$) (Chang and Randle, 2006; Zhao et al., 2005). Some studies demonstrate that selenate (SeO_4^{2-}) is the predominant Se form in neutral pH soils and represents the most available Se from plants (Gissel-Nielsen et al., 1984). Under redox conditions and lower pH, the presence of organic acids can convert selenate to selenite (SeO_3^{2-}, which is less available and mobile because it is bound onto the surface of soil particles in the dependence of soil type (Balistrieri and Chao, 1987; Johnsson, 1991; Neal et al., 1987; Pezzarossa et al., 1999; Spackman et al., 1994). The most suitable pH for Se utilization in field experiments proved to be pH 5.3, but then pH 5.2 and 5.9 demonstrate the lowest Se utilization; but a very important factor for Se up-take is soil type. The highest Se plant bioavailability can be expected in mineral soils with increasing pH (Eich-Greatorex et al., 2007). The presence of organic matter and acids, such as oxalate and citrate, inhibited the absorption of selenate (Gustafsson and Johnsson, 1992; Johnsson, 1991; Wijnja and Schulthess, 2000), but some works contradict these results (Eich-Greatorex et al., 2007). It is presumptive that soil microorganisms and mycorhizzal fungi (*Glomus* ssp.) also play a very important role in Se bioavailability due to their ability to reduce Se ions to low valence states that can consequently be incorporated into low molecular-weight complexes with

humic acids (Gustafsson and Johnsson, 1992; Larsen et al., 2006). Plants are also able to take up organic selenium forms such as Se methionine (Abrams et al., 1990b), but application of Se organic forms does not represent higher Se bioavailability (Eich-Greatorex et al., 2007). Selenium forms determines the metabolic pathway, translocation, and accumulation in plant tissues (Zayed et al., 1998b).

There are still many unanswered questions about the role of Selenium in plants. Data about Se incorporation into glutathione peroxidase are still incomplete and missing in comparison with bacteria or animals (Eshdat et al., 1997). Some plants are able to thrive in the presence of high Se concentrations, while others demonstrate growth depression and eventually death (Fu et al., 2002). Transport mechanisms of Se as selenate are connected with the transport of sulphate, and are supplied by sulphur transporter(s) (Cruz-Jimenez et al., 2005; Severi, 2001; White et al., 2007; Wu et al., 2003). It was demonstrated that selenate, as well as sulphate, are transported across plasma membranes of rhizodermal cells against their electrochemical gradient with cotransport of three protons (Hawkesford et al., 1993). First sulphate transporter genes were identified in yeasts, and later were found in higher plants, especially in rhizodermal and cortex root cells (Shibagaki et al., 2002; Smith et al., 1997, 1995; Takahashi et al., 1997). Se hyper-accumulators have a bigger ability to preferentially absorb Se over S (Ferrari and Renosto, 1972; White et al., 2004). The expression of sulphate transporter genes is regulated by a current sulphur and glutathione status in plants (Hirai et al., 2003; Maruyama-Nakashita et al., 2003). Transport of selenite is probably passive through passive diffusion; selenite up-take is readily reduced to organic Se compounds and probably in small amounts is oxidized to selenate. New studies demonstrate that selenite up-take can be connected with aquaporin activity, and this Se form can enter roots as H_2SeO_3 (Lianghe et al., 2006). Selenite transport can be inhibited by phosphates (Abrams et al., 1990a), as well as $HgCl_2$ and low temperatures (Kahakachchi et al., 2004; Lianghe et al., 2006; Shrift and Ulrich, 1969). Conversion of selenite to other forms takes place in roots (Gissel-Nielsen, 1979; Zayed et al., 1998b). It was demonstrated that the ability of certain plants to incorporate Se instead of S atoms into amino acids cysteine and methionone, and form nonproteinogenic selenoamino acids, selenocysteine (SeCys) and selenomethionine (SeMet; Fu et al., 2002; Chery et al., 2002; Neuhierl et al., 1999; Novoselov et al., 2002; Zayed et al., 1998b). This pathway plays a very important role in the plants – Se hyper-accumulators. In comparison, Se nonaccumulating plants are able to incorporate selenoamino acids into essential proteins and these substitutions cause selenium phytotoxicity (Terry et al., 2000). Selenium distribution through the plant is predominantly in selenate parallel to sulphate translocation. This Se form demonstrates the highest mobility in comparison with other selenium forms, such as selenite or organic compounds (Orser et al., 1999; Pilon-Smits et al., 1999b). Selenium tolerance, in the form of Se hyper-accumulating plants, plays a crucial role via the enzyme selenocysteine methyltransferase (Neuhierl and Bock, 1996) that is responsible for the ability to accumulate large amounts of SeCys and probably also MeSeCys (Ellis et al., 2004; LeDuc et al., 2004; Shrift and Ulrich, 1969; Sors et al., 2005), as well as the ability to reduce selenate into organic compounds. ATP suplhurylase is the next enzyme that

is probably responsible for selenium tolerance. This enzyme catalyzes the formation of both adenosine 5′-phosphosulfate (APS) and adenosine 5′-phosphoselenate (APSe) from ATP, and sulfate or selenate and its accumulation in plants (Banszky et al., 2003; Murillo and Leustek, 1995; Raspor et al., 2003), as well as APS reductase with the ability to reduce APS to selenite (Sors et al., 2005), that can then be nonenzymatically reduced by glutathione (Ng and Anderson, 1978) despite the reduction of selenodiglutathione and selenopersulfide into selenocysteine (SeCys); this pathway takes place in chloroplasts (Aketagawa and Tamura, 1980; DeSouza et al., 2000; Jablonski and Anderson, 1982; Muller et al., 1997). Cys (as well as SeCys) can enter the Met biosynthetic pathway to form Met and SeMet respectively (Lauchli, 1993), which can represent the majority of the total Se in plants (Kotrebai et al., 2000). SeMet is the main selenoamino acid in plants that are Se-sensitive (Brown and Shrift, 1981), and Se hyper-accumulators can exclude Se from SeMet and incorporate it into other selenoaminoacids and proteins like MeSeCys, SeHocys or MeSeMet (Virupaksha and Shrift, 1966). The content of selenocysteine and selenomethionine after Se treatment is highest in roots, but their rate differs in the dependence of plant species. In other vegetative plant parts, especially leaves, Se dominates in inorganic form as SeO_4^{2-} (Mounicou et al., 2006); the content of organic Se forms probably increases with the time of exposition, as shown in the study by Mazej et al. (2006) where SeMeSeCys and γ-glutamyl-SeMeSeCys were found only 41 days after exposure (Lauchli, 1993; Terry et al., 2000). The work of Pickering et al. in *Astragallus bisulcatus* shows the different distribution of individual selenium forms in leaves – in the youngest leaves, MeSeCys dominates, and in older leaves, inorganic selenate that is stored in vacuoles is dominant (Pickering et al., 2003). High Se content was also determined in seeds (Ferri et al., 2004; Smrkolj et al., 2007). SeMet can be methylated and converted to a volatile Se compound – dimethylselenide (DMSe) – in nonaccumulator plants (Tagmount and Berken, 2002), whereas some plants of *Brassica* and *Allium* genera that are able to synthesize dimethyldisulphide (DMDS) produce DMDSe from methylcysteine sulphoxide (Benevenga, 1989; Kubec et al., 1998).

Plants treated with Se demonstrate important morphological changes, such as stunted growth and a reduction in size of generative organs, especially with flowers and important changes in leaf anatomy and morphology, the absence of trichomes on leaves, and changes in leaf shape or leaf venation (Mounicou et al., 2006). Detectable changes were demonstrated in the case of Se-treated broccoli, where Se application led to a reduction in the amount of phenolic acids (cafferic, ferrulic, sinapic), as well as sulphur secondary metabolites glukosinolates (Finley et al., 2005). Selenium plays an important role as a micronutrient and has a protective effect against UV irradiation (Germ et al., 2005, 2007; Hartikainen and Xue, 1999; Pennanen et al., 2002).

One of the most utilized plants in phytoremediation is Indian mustard (*Brassica juncea*, Brassicaceae) (Montes-Bayon et al., 2002a, b); another species *Brassicaceae* is an Se hyper-accumulator – *Sinapis arvensis* (Hambuckers et al., 2008), *Brassica napus* (Banuelos et al., 1996). Very promising data provides an analysis of *Astragalus bisulcatus* and *Grindelia squarosa* with Se content in tissues

higher than 1000 mg/kg. It is obvious that these species are suitable for Se phytoextraction. The next species of genus *Astragallus* are also Se hyper-accumulators (Goodson et al., 2003). Species of genus *Mellilotus* are not considered to be Se accumulators, but some of them (e.g. *M. indica*) can accumulate more than 200 mg/kg of dry weight without growth reduction (Guo and Wu, 1998; Wu et al., 1993, 1997). Plants with a moderate ability to accumulate Se belong to the *Poaceae* family (e.g., *Agropyron, Bromus, Stipa, Festuca*) (Sharmasarkar and Vance, 2002; Wu et al., 1996). Other Se hyper-accumulators are *Larrea tridentata*, *Salvia roemeriana* (Cruiz-Jimenez et al., 2004), *Stangeria pinnata* (Goodson et al., 2003), or ferns of *Dryopteris* and *Pteris* genera (Srivastava et al., 2005). *Salicornia bigelowii* is suitable for phytovolatilization (Lin et al., 2000). Submerged parts of aquatic and wetland plants, especially shoots of *Scirpus* ssp., *Typha* ssp., *Juncus* ssp., *Myriophyllum* ssp. or *Phragmites* ssp., demonstrate the highest selenium content, and other vegetative parts such as roots intermediate and flowers and generative organs have the lowest selenium content. Species of *Typha* were determined as the most important Se hyper-accumulator (Pilon-Smits et al., 1999a; Pollard et al., 2007). In conclusion, why do plants hyper-accumulate Se? The elemental hypothesis was that detoxification and defense are the primary reasons (Hanson et al., 2004). The study of Galeas et al. (2008) brings new insight – because volatile Se forms act as a deterrent to arthropods.

4.5 Bismuth

Bismuth is a heavy metal element that chemically resembles arsenic and antimony. In the Earth's crust bismuth is about twice as abundant as gold. Bismuth occurs naturally as the metal itself and is found as crystals in the sulphide ores of nickel, cobalt, silver, and tin. The most important sources of bismuth are two ores – bismuthinite (chemically, bismuth(III) sulphide), and bismite (bismuth trioxide). Other bismuth minerals are shown in the following table.

Mineral	Chemical formula	Mineral	Chemical formula
Aikinite	$PbCuBiS_3$	Kobellite	$Pb_{22}Cu_4(Bi, Sb)_{30}S_{69}$
Berryite	$Pb_3(Ag, Cu)_5Bi_7S_{16}$	Polarite	Pd(Bi, Pb)
Bismite	Bi_2O_3	Tellurobismuthite	Bi_2Te_3
Bismuthinite	Bi_2S_3	Tetradymite	Bi_2Te_2S

Bismuth compounds generally have very low solubility and are not considered to be toxic. There is still limited information about bismuth's toxicity. The main source of environmental pollution is the smelting of copper and lead ores. Some bismuth compounds are used in medicine. Bismuth subsalicylate is still used in the therapy of gastric ulcers because of its bactericidal effects (Gilster et al., 2004; Zhang et al., 2006), while other compounds demonstrate potential cytotoxic effects (Imam, 2001; Tiekink, 2002).

Bismuth bioactive substances (BiAS) play important roles, such as the complexes of bismuth with nonionic surfactants (e.g., 4-nonylphenol, nonylphenol monoethoxylate, nonylphenol diethoxylate, and 4-tert-octylphenol) that are widely used for household and industry purposes and are formed in wastewaters. It was determined that these complexes increase bismuth solubility, as well as its up-take by plants (Fuerhacker et al., 2001). Another very important factr in bismuth up-take from contamined soils seems to be pH (Li and Thornton, 1993). The mechanisms of bismuth transport in plants in still unknown. Bismuth can interact with nuclear proteins (some staining techniques are based on bismuth compounds) (Delaespina et al., 1993), but there are some important questions – can bismuth go through plant cell walls and plasmatic membranes, and what are its interactions with the proteins of cytoplasm? Some work carried out on animal cells shows that bismuth trivalent salts increase Ca^{2+} intracellular levels as well as MAP-kinase activity and cell proliferation (Gilster et al., 2004). Studies of the bismuth effect on macrophage cell lines and human proximal tubular cells show a significant decrease of cell viability and induction of metallothioneins biosynthesis (Cowan et al., 1996; Rodilla et al., 1998; Sun et al., 1999). Another study – the influence of bismuth on tetrahymena, the group of protists – revealed extensive, dilated rough endoplasmic reticulum (rER) appearing early during the exposure as an indication of Bi-induced protein synthesis (Nilsson, 1996). There is the presumption that bismuth in more soluble forms providing up-take and transport in plants is detoxified by the same mechanisms as other heavy metals.

4.6 Other Rare Heavy Metals and Metalloids

Tellurium (Te) is a metalloid widely used in industry as semiconductor in thin films, rechargeable batteries, and charge transfer systems. Tellurium expressively affects human health. The remains of tellurium(IV) are found in water, sediment, and soil, as well as in plants (Moya et al., 2001; Suvardhan et al., 2007), and was also found in milk samples (Rodenas-Torralba et al., 2004). Tellurium up-take, transport, and metabolism are still unknown, but can probably be connected with sulphur or selenium.

Metalloid germanium (Ge) occurring in Earth's crust in trace levels is toxic to plants at high levels (Halperin et al., 1995), and to physiological functions in trace levels (Loomis and Durst, 1992) (Cakmak et al., 1995). It exhibits chemical properties similar to silicon (Si). Reciprocal ratio Ge/Si is used to trace silicon sources in marine sediments and to examine weathering processes in subtropical and tropical ecosystems (Bareille et al., 1998; Froelich et al., 1992; Kurtz et al., 2002). Whereas the role of silicon in plants is well-known (e.g., increasing mechanical strength, resistance to diseases and pests, salt, cold, heavy metal resistance, etc.) (Epstein, 1994, 1999, 2001), the role of germanium and its metabolism is still unknown. Silicon is up-taken by plants as undissociated monisilic acid (H_4SiO_4) – by passive (diffusion) as well as active transports (Raven, 1993) (Ma et al., 2006), transported from roots to aerial parts and precipitated in cell walls, intercellular spaces, and

cells as SiO2, amorphous opal (Hodson et al., 2005; Prychid et al., 2003). Silicon association with proteins were demonstrated, too (Perry and Keeling-Tucker, 2000). Data about germanium up-take and transport is compared to silicon in a very limited way, but it is obvious that are similarities to Si metabolism (Azam and Volcani, 1981; Blecker et al., 2007; Nikolic et al., 2007). A very important fact is the Ge ability to form complexes with different ligands (Pokrovski et al., 2000).

Gallium is a very rare element that occurs in nature in traces in bauxite, coal, or sphalerite, and is used in industry and as a semiconductor. Some salts, such as gallium citrate or gallium nitrate, are used as radiopharmaceutical agents used in scintigraphy; other Ga(III) salts, such as gallium maltolate, are investigated as potential anticancer drugs (Bernstein et al., 2000; Eby, 2005; Jakupec and Keppler, 2004). The antimicrobial properties of gallium were also demonstrated – it disrupts Fe up-take (Kaneko et al., 2007). In a study carried out on algae *Chara corallina*, Ga demostrated lower cytotoxic effects than aluminium. This study also compared scandium, which demonstrated the most toxic effect in comparison with Ga and Al (Reid et al., 1996). The high gallium up-take by roots was confirmed in a study by Wheeler et al (Wheeler and Power, 1995). Iron deficiency was demonstrated in plants treated with Ga(III), so the mechanism of gallium cytotoxic effect consisted of disrupting Fe up-take (Johnson and Barton, 2007); disruptions and interactions of aluminium up-take was not determined (Wheeler et al., 1993).

Scandium bioaccumulation was demonstrated on wheat seedlings, which showed better growth parameters compared to media without Sc supplementation in comparison with previous studies (Reid et al., 1996). Transfer of seedlings growing in Sc-enriched media to normal culture media led to a decrease of Sc levels in aerial parts (mainly leaves), but Sc concentration in roots remained high (Shtangeeva et al., 2004).

Plants are able to up-take gold (Au) from soil. These conclusions were confirmed in a study by Lasat on alfalfa plants that demonstrated the ability to uptake gold from media (Lasat, 2002). There are limitations on gold up-take – gold, in natural conditions, has very low solubility, and gold itself is a rare element. The supplementation of media (soil) with Au-chelating agents significantly increases the Au up-take by plants. In experiments, the cultivation media were supplemented with ammonium thiocyanate (experimental plant *Brassica juncea*) (Anderson et al., 1998) and cyanate (*Brassica juncea*, *Cichorium* ssp.) (Lamb et al., 2001). Cyanate seems to be a better gold chelator and demonstrated higher efficiency of gold up-take in comparison with thiocyanate. In comparison with other crops (red beet, onion and radish) carrot (*Daucus carota*, Apiaceae) demonstrated the highest ability to accumulate Au in field experiments. What demonstrated half that ability (Carrillo-Castaneda et al., 2002; Msuya et al., 2000). A positive effect of cyanates (cyanogenic extract from *Prunus laurocerasus*) was determined on maize plants (*Zea mays*), with the highest Au concentrations in roots (Girling et al., 1978). A very interesting study was published in 2005. The desert willow, *Chilopsis linearis*, a common inhabitant of the Mexican Chihuahua Desert (Gardea-Torresdey et al., 2005), was treated with different concentrations of ammonium thiocyanate; Au uptake was investigated, too. Ammonium thiocyanate itself showed root elongation inhibition in high

concentrations; shoots were not affected. The highest Au concentrations without ammonium thiocyanate application were found in roots – the lowest in leaves (63 mg Au/kg dry mass, 4.5 mg Au/kg dry mass, respectively). Application of ammonium thiocyanate in very low concentrations (1×10^{-5} M) led to an increse of Au concentrations in leaves, but not in shoots or roots, whereas application of ammonium thiocyanate in higher concentrations (5×10^{-5} mol/l and more) led to total Au concentration increasing with highest concentrations in roots (437 mg/kg DW). Au is absorbed and presented in plants in reduced Au(0) form (more than 90%), with only very small amounts as Au(I) and Au(III) forms. As indicated in a previous study, plants were able to up-take Au as Au(III) and then reduce it to Au(0) in root tissues. Au mobility in tissues is probably very limited. Gold hyperaccumulation is defined as containing 1 mg Au in 1 kg of dry mass. This concentration is about 100 times higher than the common concentration in Au nonaccumulating plants (Anderson et al., 1999; Gardea-Torresdey et al., 2002). Douglas fir, *Pseudotsuga menziesii*, is used to determine gold deposits in Canada (Dunn, 1995); the species of genus *Artemisia* is recommended for Au prospecting (Erdman and Olson, 1985). *Zea mays* and *Brassica juncea* are considered to be Au bioaccumulators after cyanide treatment (Anderson et al., 2005). One interesting conclusion – flower antheral cells can be used as surface modifiers due to their ability to bioaccumulate Au in connection with electrochemical measurements for gold preconcentration before their own measurement (Wang et al., 1992).

Platinum, together with palladium (Pd), rhodium (Rh), ruthenium (Ru), iridium (Ir), and osmium (Os), are considered platinum group metals (PGM). They are widely used in industry (automobile catalytic converters) and especially in oncology because of their ability to influence the cell cycle as well as process cell division. Pd, Pt, and Rh are released from catalytic converters and tey form emissions as exhaust fumes. They are then deposited along the roads, on vegetation, and in soils (Jankowski, 1995; Niemela et al., 2004; Wei and Morrison, 1994). They can also accumulate in water and affect aquatic environments. Their chemistry makes them less amenable for phytoremediation. Higher PGM (rhenium) bioavailability was observed in rice paddy fields; soluble Rh forms (together with technetium) are considered to be highly bioavailable (Tagami and Uchida, 2004, 2008). Ruthenium bioaccumulation was investigated in *Lemna* tissues and was determined to be very low compared to cesium (Polar and Bayulgen, 1991). The highest ruthenium bioaccumulation was observed in the case of Geraniales and Asterales orders – the lowest in Poales (Willey and Fawcet, 2006). The ability of *Citrobacter* sp. to bioaccumulate Pd was demonstrated (Yong et al., 2002). *Desulfovibrio desulphuricans* has the ability to bioaccumulate Pd(II) as chloro- and aminocomplexes specifically in the presence of Pt(IV) and Rh(III) up to 15% of dry weight and its consequent bioreduction into a Pd(0)-suitable model for Pd bioaccumulation (Yong et al., 2002). Also other sulphate reducing bacteria are suitable for PGM accumulation. Some aquatic organisms, such as isopod *Asellus aquaticus,* occur in polluted waters as well as clean waters, so they can serve as model organisms in heavy metal investigations (Murphy and Learner, 1982). The accumulation of heavy metals in freshwater isopods was demonstrated in many studies (Mulliss et al., 1994; VanHatum et al., 1993), but

the isopods were also able to accumulate PGM (Moldovan et al., 2001). The number of studies interested in PGM up-take and distribution in plants is very limited. The ability of some plants to up-take PGM was documented in grasses and cucumbers. *Sinapis alba* and *Lolium perenne* demonstrated Pt up-take. A very important feature of PGM is their ability to form complexes. Platinum was translocated from roots to all aerial plant organs, but maximum content was detected in roots. The mechanism of this absorption, as well as binding and metabolism, is still unknown. More than 90 % of absorbed Pt was found in molecular fractions lower than 10 kDa bound to ligand-carbohydrates, not peptides (Alt et al., 1998), which were determined as oligosaccharides of 2–5 monomeric units of aldonic, aldaric, and uronic acids that can originate from pectin hydrolysis (Alt et al., 1998). The Pt effect on plant-cell models was investigated on tobacco BY-2 suspension culture by Babula et al. The ability of platinum as cisplatin to affect the processes of mitosis leading to programmed cell death were determined (Babula et al., 2007). Some species, such as *Lolium* ssp. and *Sinapis* ssp., are considered to be suitable Pt bioindicators, especially in their ability to grow alongside roads (Kologziej et al., 2007; Kowalska et al., 2004).

Technetium (Tc) is a nonessential element that can be absorbed by plants, as shown in a study using tomato plants. The most suitable Tc form is TcO_4^-. Microorganisms as well as redox soil potential play important role in Tc state in soil (Tagami and Uchida, 1996). Reduced Tc forms, such as Tc(IV), are bound to soil particles and because of this are less bioavailable (Ashworth and Shaw, 2005). The role of anion transport proteins is still being discussed (Tagami and Uchida, 2005). After TcO_4^- transports across root surfaces, it probably moves through cells and xylem to all plant aerial organs. Different Tc forms were found in tissues: TcO_4^-, Tc-cys, and Tc-glutathione (Krijger et al., 1999; Lambtechts et al., 1985). The highest Tc concentrations were found in leaves (Mousny et al., 1979). These results confirm the theory that Tc can be reduced in tissues to organic compounds (Krijger et al., 1999; Simonoff et al., 2003). Tc itself is highly accumulated in plants. Tc application in wide concentration ranges do not cause symptoms of toxicity (Mousny et al., 1979).

Tungsten (W) is a widely used metal, especially in industry and the military. The potential effect of tungsten on the environment is still unknown. Some results show that this element can be introduced into the food chain (Strigul et al., 2005). Tungsten itself has the capacity to influence plant growth; the application of tungsten to cultivation media led to the inhibition of root nitrate reductase activity and NO_3^- uptake in sunflower plants without affecting nitrite reductase activity (DeLaHaba et al., 1990). The work of Notton et al. using spinach plants (*Spinacea oleracea*) demonstrated the ability of W to form an analogue of nitrate reductase without activity (Notton and Hewitt, 1971, 1974). This effect led to growth depression and plant death. The plants bioaccumulating tungsten are *Digitalis purpurea*, *Chamaespartium tridentatum*, *Cistus ladanifer*, *Pinus pinaster*, *Erica umbellata*, and *Quercus ilex* subsp. *ballota* (Pratas et al., 2005).

Uranium (U) and thorium (Th) are naturally occurring radioactive elements widely distributed in the lithosphere as well as stratosphere. Their content in soils depends especially upon geological conditions, but may be influenced by nuclear

accidents (Xu et al., 2002a). Uranium up-take is highly dependent on soil pH (Ebbs et al., 1998) and depends on the content of organic compounds in soil because of their ability to influence the mobility of U in soil (Bednar et al., 2007). The most mobile are U(VI) salts, as predominantly UO_2^{2+} (Grenthe et al., 1992) and carbonate complexes (Duff and Amrhein, 1996); other forms are less bioavailable and remain in bound to soil particles. Mycorrhizal fungi (especielly of *Glomus* genus) can significantly increase U up-take (Rufyikiri et al., 2004, 2003, 2002) due to their capacity for heavy metals (Chen et al., 2005) and the ability to enhance U immobilization. Fungal mycelium probably can help translocate U as uranyl cations toward roots through fungal tissues (Rufyikiri et al., 2002; Weiersbye et al., 1999). These results were confirmed on experimental plants of *Medicago trunctula* cv. Jemalong by comparising U treatment with and without the presence of mycorrhizal fungus *Glomus intraradices* (Baodong et al., 2005). Experimental plants associated with this fungus demonstrate higher U uptake and U content in roots. Uranium concentrations in stems were higher in inoculated plants, so it means that U was entranced by mycorrhizal root colonization. Phytoextraction (up-take) of U can also be induced by organic acids, especially citric acid (Huang et al., 1998); U and Th up-take is also probably associated with iron content in plants (Rodriguez et al., 2002) and with their ontogenetic state (Singh et al., 2005). In plant tissues, uranium is probably bound as uranyl(VI) phosphate to phosphoryl groups (Gunther et al., 2003). Experiments were carried out on the plants of *Capsium annuum* and *Cucumis sativus*, which were treated by nitrate salts of uranium and thorium and demonstrated a wide range of growth anomalies, especially in the case of uranium. The highest U and Th contents were determined in stems. The same concentrations of thorium affected growth of experimental plants less expressively, and these plants also demonstrated higher vitality (Unak et al., 2007). It is obvious that plants are able to up-take these elements in forms that are soluble in water, and they are distributed from roots to aerial parts. There are only a few plants with U bioaccumulation ability (Whiting et al., 2004), such as *Uncinia leptostachya, Coprosma arborea*, with U contents in tissues higher than 3 mg/kg of dry mass (Peterson, 1971). The highest U content in the needles of *Picea mariana* was determined as more as 1000 mg/kg of dry mass.

The rare earth elements (REEs) form a chemically uniform group and include yttrium (Y), lanthanum (La), and 14 lanthanides: cerium (Ce), dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), holmium (Ho), lutetium (Lu), neodymium (Nd), promethium (Pm), praseodymium (Pr), samarium (Sm), terbium (Tb), thulium (Tm), and ytterbium (Yb). Some of them occur in rocks, such as granites or pegmatites, as phosphates, carbonates, silicates, or fluorides (Bauluz et al., 2000; Buhn et al., 2002; Masau et al., 2000). Some studies prove anthropogenic origins of some REEs, especially because of their utilization in medicine in imaging methods (Ogata and Terakado, 2006). They usually form trivalent, rarely tetravalent, or divalent cations. Their status in soil, as well as their mobility, depends upon pH (pH plays an important role in the stability of complexes), organic matter (negative roles in bioavailability as chelating and adsorbing agents), and clay minerals content (Price et al., 1991; Wu et al., 2001b; Wu et al., 2001c). Soil microorganisms play an important role in the availability of REEs with their biosorption ability (Andres

et al., 2000) and production of organic acids that can serve as solubilizers of phosphates, especially (Schijf and Byrne, 2001).

Some REEs may promote growth, plant development, and crop production in very low concentrations, but our knowledge is still very limited (Diatloff et al., 1999; Huang et al., 2003; Shi et al., 2006; Shtangeeva and Ayrault, 2007; Wen et al., 2001; Xie et al., 2003; Xiong et al., 2006; Xu et al., 2002b). Many experiments are carried out in China, where some studies on rice (*Oryza sativa*) proved the Lanthan ability to increase growth, dry root weight, and grain numbers (Xie et al., 2002), as well as drought tolerance (Xiong et al., 2005) or germination ratio (Hong et al., 2003). What is the explanation of these effects? Some lanthanoids are able to replace endogenous calcium ions in some enzymes, such as peroxidase in horseradish (Morishima et al., 1986) or calmodulin in pea seedlings (Amann et al., 1992) and *Amaranthus caudatus* seedlings (Zeng et al., 2003) with retaining its activity. In addition, Nd(III) is able to replace Ca(II) under conditions of Ca deficiency (Wei et al., 2001; Zhang and Shan, 2001). La(III), as well as Ce(III), seems to be effective in floral induction; La(III) increases the rate of photosynthesis in experimental condions by promoting Mg-ATPase and activation of ribulose biphosphate carboxylase (Chen et al., 2000, 2001). All these results show the REE's ability to replace Ca(II) in the physiological processes of plants (Zeng et al., 2000). Increasing photosynthetic activity by REEs was also demonstrated on fern *Dicranopteris dichotoma*. This fern grows in acid soils (pH 4−5) and can hyper-accumulate more than 0.1% La, Ce, Pr and Nd in leaves (w/w of dry mass). These REEs were identified in membranes of chloroplasts, as well as thylakoids, probably due to their ability to bind to membranes and photosystem II proteins (more than 25% of REEs chloroplast content). Negative carboxyl and hydroxyl groups may chelate and bind these rare elements (Wang et al., 2003). REEs are probably able to bind to chlorophyll and replace Mg(II) ions; $[\text{Chl-a-La-pheophytin}]^{2+}$ complex was identified in spinach leaves after La application; acceleration of light energy transformation to electric energy, the electron transport, water photolysis, and oxygen evolution of PSII of spinach was demonstrated, too (Hong et al., 2005b). This effect was also shown in spinach leaves after Ce(III) treatment, when Ce-chlorophyll originated (Liu et al., 2007). Chlorophyll content, as well as photosynthetic rate, was increased (Hong et al., 2002, 1999). The next possible effect of REEs mechanism of action is in a role as metallic-activated factors of certain enzymes, which catalyze the catabolism and anabolism of proteins (Hong et al., 2005a; Tian et al., 2005), and increasing activity of enzymes in the reactive oxygen species scavenging system (Xiao et al., 2007; Yan et al., 2007; Zhang et al., 2003). Negative REEs effects on plants occur in higher concentrations.

The mechanisms of uptake are not fully clarified, but all REEs may be reduced to a divalent state (Tian et al., 2003); trivalent cations are probably able to migrate in plant bodies as it was demonstrated in the case of cerium(III) in horseradish (Guo et al., 2007). A rare earth element-binding protein, unlikely to be phytochellatine, was isolated from maize. Its molecular weight is 183.000 and consists of two subunits. This protein is rich in asparagine/aspartic acid, glutamine/glutamic acid, glycine, alanine, and leucine, and contains 8.0% of covalently bound carbohydrates

(Yuan et al., 2001). A concentration of REEs is generally highest in roots, in comparison with stems and leaves in vascular plants, and the lowest content was identified in fruits and seeds; higher REE content was also determined in plant tops (Xu et al., 2003), and an accumulation of REEs may also be connected with the ontogenetic state of plants (Wen et al., 2001; Wyttenbach et al., 1994; Xu et al., 2003). Experiments with rice and peas show limited distribution of La and Yb to the xylem and endodermis (Ishikawa et al., 1996). In some tropical trees, Ce was accumulated in bark (Nakanishi et al., 1997). Foliar application of REE solutions led to their distribution to stems and roots (Wang et al., 2001). Lanthanoids in in vitro systems increased the secondary metabolite production (Wu et al., 2001a).

Several ferns are known as accumulators of REEs. Genera *Dryopteris, Asplenium, Adianthum* and *Dicranopteris* demonstrate bioaccumulation of La and Ce (Ozaki et al., 2002, 2000) together with Y (Wang et al., 1997); the highest REE amount was localized in root surfaces in precipitated form and cortical cells. In mosses and lichens, *Hypogymnia physodes* was identified as an lanthanoid accumulator (Markert, 1987; Markert and Deli, 1991). Information about the up-take of other elements, as well as the distribution and metabolism in plants, are very limited and still unavailable.

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Role of Plant Growth Promoting Rhizobacteria in the Remediation of Metal Contaminated Soils: A Review

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Abstract Pollution of the biosphere by toxic metals is a global threat that has accelerated dramatically since the beginning of the industrial revolution. The primary source of metal pollution includes industrial operations such as mining, smelting, metal forging, combustion of fossil fuels, and sewage sludge application in agronomic practices. The metals released from these sources accumulate in the soil and, in turn, adversely affect the microbial population density and the physico-chemical properties of soils, leading to the loss of soil fertility and crops yields. The heavy metals can not generally be biologically degraded to more or less toxic products, and hence persist in the environment. Conventional methods used for metal detoxification produce large quantities of toxic products and are cost-effective. The advent of bioremediation has provided an alternative to conventional methods for remediating metal-poisoned soils. In metal-contaminated soils, the natural role of metal-tolerant plant growth promoting rhizobacteria to maintain soil fertility is more important than in conventional agriculture, where greater use of agrochemicals minimizes their significance. Besides their role in metal detoxification and removal, rhizobacteria also promotes the growth of plants by other mechanisms, such as the production of growth-promoting substances and siderophores. Phytoremediation is another emerging low-cost, in situ technology employed to remove pollutants from contaminated soils. The efficiency of phytoremediation can be enhanced by the judicious and careful application of appropriate heavy metal-tolerant, plant growth-promoting rhizobacteria, including symbiotic nitrogen-fixing organisms. This review presents the results of studies on the recent developments in the utilization of plant growth-promoting rhizobacteria for direct application in soils contaminated with heavy metals under a wide range of agroecological conditions, with a view to restoring contaminated soils and, consequently, promoting crop productivity in metal-polluted soils across the globe and their significance in phytoremediation.

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1 Introduction

The release of heavy metals from various industrial sources, agrochemicals, and sewage sludge present a major threat to the soil environment. Generally, heavy metals are not degraded biologically and persist in the environment indefinately. Once accumulated in the soils, the toxic metals affect the microbial compositions, including plant growth-promoting rhizobacteria (PGPR) in the rhizosphere, and their metabolic activities. In addition, the elevated concentration of metals in soils, and their uptake by plants, adversely affect the growth, symbiosis, and consequently yield of crops (Moftah, 2000; Wani et al., 2007a, 2008a) by disintegrating cell organelles and disrupting membranes (Stresty and Madhava, 1999); acting as a genotoxic substance (Sharma and Talukdar, 1987); disrupting the physiological process, such as photosynthesis (Van Assche and Clijstersters, 1990; Wani et al., 2007b), or by inactivating the respiration, protein synthesis, and carbohydrate metabolism (Shakolnik, 1984). The remediation of metal-contaminated soils thus becomes important, as these soils usually cover large areas that are rendered unsuitable for sustainable agriculture.

To circumvent the metal stress microorganisms of agronomic importance have evolved a number of mechanisms that they use to tolerate the uptake of heavy metal ions. Such mechanisms include: (i) the pumping of external metal ions to the cell, (ii) the accumulation and sequesteration of the metal ions inside the cell, and (iii) the transformation of toxic metal to less toxic forms (Wani et al., 2008b) and adsorp-

tion/desorption of metals (Mamaril et al., 1997). Due to these properties, when PGPR, including nitrogen fixers used as seed inoculants, are applied to soil, either treated intentionally with metals or already contaminated, they have resulted in a substantial reduction in the toxicity of metals and concomitantly improved the overall growth and yield of chickpea (*Cicer arietinum*) (Gupta et al., 2004), greengram (*Vigna radiata* L. wilczek) (Wani et al., 2007a), and pea (*Pisum sativum*) (Wani et al., 2007c). Besides their role in protecting the plants from metal toxicity, the PGPR are also well-known for their role in enhancing soil fertility and promoting crop productivity by providing essential nutrients (Zaidi et al., 2003, 2004; Zaidi and Khan, 2006) and growth regulators (Wani et al., 2007d, e). They also promote the growth of plants by alleviating the stress induced by ethylene-mediated impact on plants (Glick et al., 2002) by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Uchiumi et al., 2004; Belimov et al., 2005). Use of such microbes possessing multiple properties of metal resistance and an ability to promote plant growth through different mechanisms in metal-contaminated soils make them one of the most suitable choices for bioremediation studies.

The other alternative approach used to clean up the contaminated soils using plants is the innovative technique known as phytoremediation (Brooks, 1998). This technology involves the use of metal-accumulating plants to remove, transfer, or stabilize the contaminants from soils, but this technique is time consuming (Wenzel et al., 1999). The efficiency of the phytoremediation technique is, however, influenced by the activity of rhizosphere microbes and the speciation and concentration of metals deposited into the soil (Wang et al., 1989; Khan, 2005b). For instance, the use of PGPR *Pseudomonad* and *Acinetobacter* has been shown to enhance the phytoremediation abilities of nonhyperaccumulating maize (*Zea mays* L.) plants by increasing their growth and biomass (Lippmann et al., 1995). Also, plants growing in metal-stressed soils can protect itself from metal toxicity by synthesizing antioxidant enzymes, which scavenge the toxicity of reactive oxygen species generated by plants (Cardoso et al., 2005) and by associative bacteria (Corticeiro et al., 2006) under metal stress.

2 Growth Promotion by Plant Growth-Promoting Rhizobacteria (PGPR)

The rhizosphere bacteria capable of aggressively colonizing plant roots and promoting plant growth are generally called plant growth-promoting rhizobacteria (Kloepper and Schroth, 1978). Broadly, PGPR can be divided into two major groups according to their relationship with the host plants: (i) symbiotic rhizobacteria, and (ii) free-living rhizobacteria (Khan, 2005b) that can invade the interior of cells and survive inside (intracellular PGPR, such as nodule bacteria) or remain outside the plant cells (extracellular PGPR, such as *Bacillus, Pseudomonas, Azotobacter,* etc.). These organisms affect plant growth in three different ways: (i) by synthesizing and providing particular compounds to the plants (Glick, 1995), (ii) facilitating the uptake of certain nutrients from environment (Çakmakçi et al., 2006),

and (iii) protecting plants from certain diseases (Khan et al., 2002). Rhizobacteria generally improves plant growth by synthesizing phytohormones precursors (Perveen et al., 2002; Ahmad et al., 2008), vitamins, enzymes, siderophores, antibiotics (Burd et al., 2000; Glick, 2001), and inhibiting ethylene synthesis. In addition, the rhizobacterial strains can solubilize inorganic P (Zaidi and Khan, 2005, 2007; Khan and Zaidi, 2007), mineralize organic P (Ponmurugan and Gopi, 2006; Khan et al., 2007), and improve plant-stress tolerance to drought, salinity, and metal toxicity, leading thereby to increased plant growth. The growth-promoting substances synthesized by various rhizobacteria are summarized in Table 1.

Moreover, the PGPR also increase the growth of plants through the synthesis of specific enzymes, which induce physiological changes in plants. For example, ethylene plays a critical role in various developmental processes, such as leaf senescence, leaf abscission, epinasty, and fruit ripening (Vogel et al., 1998). Ethylene also regulates nod-factor signaling and nodule formation and has primary functions in plant defense systems. Moreover, ethylene production increases as a result of plant infection by rhizobacteria (Schmidt et al., 1999). At higher concentrations, ethylene inhibits the growth and development of plants (Grichko and Glick, 2001). However, bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase, synthesized by PGPR (Belimov et al., 2005; Safronova et al., 2006; Madhaiyan et al., 2006; Rajkumar et al., 2006) alleviates the stress induced by the ethylene-mediated impact on plants (Glick et al., 2002). The ACC of roots is metabolized by ACC deaminase into ketobutyrate and ammonia (Penrose and Glick, 2001). The bacteria utilize NH_3 evolved from ACC as a source of N, and thereby restrict the accumulation of ethylene within the plant, which otherwise inhibits plant growth (Yang and Hoffman, 1986; Belimov et al., 2002).

Among other PGPR, the symbiotic nitrogen fixers enhance the growth of legumes by: (i) providing N to the plants through N_2 fixation (Zaidi et al., 2004), (ii) increasing the availability of nutrients in the rhizosphere, (iii) inducing increases in root surface area, (iv) enhancing other beneficial symbioses of the host, (v) reducing or preventing the deleterious effects of phytopathogenic organisms (Khan et al., 2002), and (vi) combination of modes of action. As an example of a plant-growth promoter, indoleacetic acid (IAA), a phytohormone of the auxin series produced by many rhizobia (Abd-Alla, 1994; Wani et al., 2007f, g, 2008b), and its metabolically related precursor, anthranilic acid, can reductively solubilize soil Fe (III), and increase its availability via a mechanism different from that involving siderophores (Kamnev, 1998; Kamnev et al., 1999). In a study, Leinhos and Bergmann (1995) and Lippmann et al. (1995) reported that the addition of IAA to soil enhanced the uptake of iron and other elements (e.g., zinc, calcium, etc.) in plant roots.

Another growth-promoting substance, the siderophore, is a specific Fe (III)-chelating agent that makes the chelated iron unavailable to pathogenic microorganisms (Braun, 1997), and leads to an increase in plant health. Microbial siderophores are known to regulate the availability of Fe in the plant rhizosphere (Loper and Henkels, 1999), and it has been found that competition for iron in the rhizosphere is controlled by the affinity of the siderophores for iron. Interestingly, the binding affinity of phytosiderophores for iron is less than the affinity of microbial siderophores,

Table 1 Growth regulators produced by PGPR

Organisms	Growth regulators	Reference
Azotobacter,Fluorescent pseudomona, and *Bacillus*	IAA, Siderophore, Ammonia, HCN, P-solubilization	Ahmad et al. (2008)
Bacillus spp.	IAA, P solubilization, Siderophores, HCN, ammonia	Wani et al. (2007d)
Bacillus spp.	IAA, P solubilization, Siderophores, HCN, ammonia	Wani et al. (2007e)
Azotobacter chroococcum	IAA, Siderophores, HCN, ammonia	Wani et al. (2007d)
Pseudomonas and *Bacillus*	Siderophores, IAA, P-solubilization	Rajkumar et al. (2006)
Brevibacillus sp.	IAA	Vivas et al. (2006)
Bravibacterium sp.	Siderophore	Noordman et al. (2006)
Xanthomonas sp. *RJ3, Azomonas sp. RJ4,Pseudomonas sp. RJ10, Bacillus sp. RJ31*	IAA	Sheng and Xia (2006)
Bacillus subtilis	IAA and P-solubilization	Zaidi et al. (2006)
Bacillus sp.	P-solubilization	Canbolat et al. (2006)
Variovorax paradoxus, *Rhodococcus* sp. and *Flavobacterium* (Cd tolerant)	IAA and siderophores	Belimov et al. (2005)
Kluyvera ascorbata	Siderophore	Burd et al. (2000)
Pseudomonas fluorescens	IAA, siderophore and P-solubilization	Gupta et al. (2005)
Pseudomonas putida	Siderophore	Tripathi et al. (2005)
Sphingomonas sp, *Mycobacterium* sp, *Bacillus* sp, *Rhodococcus* sp, *Cellulomonas* sp. and *Pseudomonas* sp.	IAA	Tsavkelova et al. (2005)
*Azotobacter,*Fluorescen tp*seudomonas*	IAA	Ahmad et al. (2005)
Seratia spp, Pseudomonas spp and Bacillus spp.	IAA, P-solubilization	Wani et al. (2005)
Bacillus and *Azospirillum* sp.	IAA, P-solubilization	Yasmin et al. (2004)
Micrococcus luteus	IAA, P-solubilization	Antoun et al. (2004)
Bacillus, Pseudomons, Azotobacter, and Azospirillum	P-solubilization and IAA	Tank and Saraf (2003)
Pseudomonas sp.	IAA, siderophore and P-solubilization	Gupta et al. (2002)
Pseudomonas fluorescence	Siderophore	Khan et al. (2002)
Azotobacter chroococcum	Gibberellin, kinetin, IAA	Verma et al. (2001)
Azotobacter chroococcum	P-solubilization	Kumar et al. (2001)

but plants require a lower iron concentration for normal growth than do microbes (Meyer, 2000). However, several rhizobial species are known to produce growth-promoting substances in metal-free environments, but the synthesis of these compounds by metal-tolerant rhizobia are limited. Nevertheless, there has been certain evidence where metals at lower concentrations either exert no harmful effect

Table 2 Plant growth-promoting substances produced by symbiotic nitrogen fixers both in metal-free and metal-stressed environments

Symbiotic N_2fixers	Heavy metal	Plant growth promoting substances	References
Rhizobium, *Bradyrhizobium*	–	P-solubilization	Abd-Alla (1994)
Bradyrhizobium japonicum	–	Siderophore	Wittenberg et al. (1996)
Rhizobium leguminosarum	–	Cytokinin	Noel et al. (1996)
Rhizobium ciceri	–	Siderophopre	Berraho et al. (1997)
Bradyrhizobium, Rhizobium	–	Siderophore	Duhan et al. (1998)
Bradyrhizobium, Rhizobium	–	IAA	Antoun et al. (1998)
Rhizobium meliloti	–	Siderophore	Arora et al. (2001)
Mesorhizobium, Bradyrhizobium sp. (*vigna*)	–	Siderophore	Khan et al. (2002)
Rhizobium	–	HCN, siderophore	Deshwal et al. (2003)
Bradyrhizobium (Arachis)	–	Siderophore, IAA and P-solubilization	Deshwal et al. (2003)
Rhizobium	–	P-solubilization and IAA	Tank and Saraf (2003)
Bradyrhizobium, *Rhizobium*	–	IAA, P-solubilization	Antoun et al. (2004)
Bradyrhizobium japonicum	–	IAA	Shaharoona et al. (2006)
Bradyrhizobium sp. RM8	Nickel, Zinc	IAA, Siderophore, Ammonia, HCN	Wani et.al. (2007f)
Rhizobium sp. RP5	Nickel, Zinc	IAA, Siderophore	Wani et.al. (2007c)
Rhizobium sp. RL9	Zinc	IAA, Siderophore, Ammonia, HCN	Wani et.al. (2007 g)
Mesorhizobium sp. RC3	Chromium (vi)	IAA, Siderophore	Wani et.al. (2008b)
Mesorhizobium	–	IAA, Siderophore, Ammonia, HCN, P-solubilization	Ahmad et al. (2008)

on the rhizobia or even stimulate plant growth-promoting activities. For instance, *Bradyrhizobium* strain RM8, tolerant of nickel and zinc, *Rhizobium* sp. RL9, isolated from lentil nodules and tolerant to zinc, and *Rhizobium* sp. RP5, isolated from pea nodules and tolerant to zinc and nickel, produced substantial amounts of IAA (Wani et. al., 2007f, g, c). The production of growth promoting substances by metal-tolerant and natural rhizobial strains are presented in Table 2.

3 Biological Availability of Metals in Soil

Heavy metals such as lead, arsenic, cadmium, copper, zinc, nickel, and mercury are discharged from industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, and combustion of fossil fuel. Moreover, agricultural activities like application of agrochemicals and long-term usage of sewage sludge in agricultural practices also adds a significant amount of metals to the soil (Giller et al., 1989; McGrath et al., 1995). These metals exists in bioavailable and nonbioavailable forms (Sposito, 2000) whose mobility depends on two factors: (i) the metallic element that precipitates as positively charged ions (cations), and (ii) the element that makes up the negatively charged component of salt.

Physicochemical properties of soils, such as cation exchange capacity (CEC), organic matter, clay minerals and hydrous metal oxides, pH and buffering capacity, redox potential and extent of aeration, water content and temperature together with root exudates and microbial activities, determines the metal availability in soils (Brown et al., 1999; Traina and Laperche, 1999; Krishnamurthy, 2000). The toxicity of metals within soils with high CEC is generally low, even at high total metal concentration (Roane and Pepper, 2000). Under oxidized and aerobic conditions, metals are usually found in soluble cationic forms while in reduced or anaerobic states, as sulphide or carbonate precipitates. In low-pH soil, the metal bioavailability increases due to its free ionic species while at high-pH soil it decreases due to insoluble metal mineral phosphate and carbonate formation. The mobility and bioavailability of certain metals in soils is usually in the order: Zn $>$ Cu $>$ Cd $>$ Ni (Lena and Rao, 1997). However, the concentration of heavy metals within all components of the ecosystem varies considerably.

Coexistence and persistence of metals in soils as multiple contaminants facilitate the entry and accumulation of these pollutants into food webs and ultimately into human diets. Contamination of agronomic soils with heavy metals has thus become a global threat to the sustainability of the agro-ecosystems and, therefore, is receiving considerable attention from environmentalists. Therefore, the assessment of heavy metal bioavailability and the uptake of metals by plants help in: (i) evaluating the impact of metals on beneficial rhizospheric microbes and crops grown in metal stressed soils, and (ii) predicting the application of bioremediation technologies that could be used to clean up metals from the polluted soils. The remediation of such soils, therefore, requires urgent attention so that the sustainability of crops and, in turn, food security across the globe can be protected.

4 How Plant Growth-Promoting Rhizobacteria Combat Heavy Metal Stress

Accumulation of heavy metals in the soil environment and their utake by both PGPR and plants is a matter of growing environmental concern. Unlike many other pollutants, which can undergo biodegradation and produce less toxic, less mobile, and less bioavailable products, heavy metals are difficult to remove from contaminated environments. These metals cannot be degraded biologically, and are ultimately indestructible, although the speciation and bioavailability of metals may change with variation in environmental factors. Some metals, such as zinc, copper, nickel, and chromium are essential or beneficial micronutrients for plants, animals, and microorganisms (Olson et al., 2001), and others (e.g., cadmium, mercury and lead) have no known biological and/or physiological functions (Gadd, 1992). However, the higher concentration of these metals has great effects on the microbial communities in soils in several ways: (i) it may lead to a reduction of total microbial biomass (Giller et al., 1998), (ii) it decreases the numbers of specific populations (Chaudri et al., 1993), or (iii) it may change microbial community structure (Gray and Smith, 2005). Thus, at high concentrations, metal ions can either completely inhibit the microbial population by inhibiting their various metabolic activities (Fig. 1), or organisms can develop resistance to or tolerance of the elevated levels of metals.

The ability to grow even in high metal concentration is found in many rhizospheric microorganisms including symbiotic N_2-fixing bacteria (Lakzian et al., 2002), and may be the result of intrinsic or induced mechanisms (Giller et al., 1998). Tolerance may be defined as the ability to cope with metal toxicity by means of intrinsic properties of the microorganisms, while resistance is the ability of microbes to survive in higher concentrations of toxic metals by detoxification mechanisms, activated in direct response to the presence of heavy metals (Ledin, 2000). Toxic heavy metals, therefore, need to be completely removed from the contaminated soil,

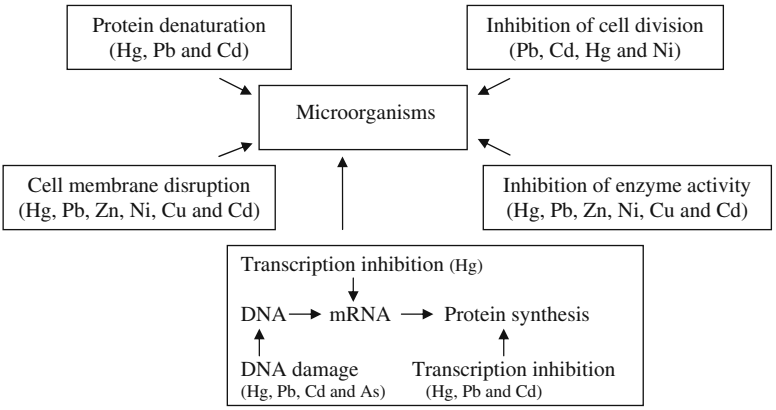

Fig. 1 Heavy metal toxicity mechanisms for microbes

transformed, or immobilized, to produce much less or nontoxic species. However, to survive and proliferate in metal-contaminated soils, tolerance has to be present both in microbes and their associative hosts.

For survival in a metal-stressed environment, PGPR have evolved several mechanisms by which they can immobilize, mobilize, or transform metals, rendering them inactive to tolerate the uptake of heavy metal ions (Nies, 1999). These mechanisms include: (i) exclusion—the metal ions are kept away from the target sites; (ii) extrusion—the metals are pushed out of the cell through chromosomal/plasmid mediated events; (iii) accommodation—metals form complexes with the metal-binding proteins (e.g., metallothienins, low molecular weight proteins) (Kao et al., 2006; Umrania, 2006) or other cell components; (iv) biotransformation—toxic metal is reduced to less toxic forms; and (v) methylation and demethylation. One or more of these defense mechanisms allow these microorganisms to function metabolically in an environment polluted by metals. These mechanisms could be constitutive or inducible.

The bacterial resistance mechanisms are generally encoded on plasmids and transposons, and it is probably by gene transfer or spontaneous mutation that bacteria acquire their resistance to heavy metals. For example, in Gram-negative bacteria (e.g., *Ralstonia eutropha*), the *czc* system is responsible for the resistance to cadmium, zinc, and cobalt. The *czc* genes encode for a cation-proton antiporter (CzcABC), which exports these metals (Nies, 1996). A similar mechanism, called the *ncc* system, has been found in *Alcaligenes xylosoxidans*, which provides resistance against nickel, cadmium, and cobalt. On the contrary, the cadmium resistance mechanism in Gram-positive bacteria (e.g., *Staphylococcus*, *Bacillus*, or *Listeria)*

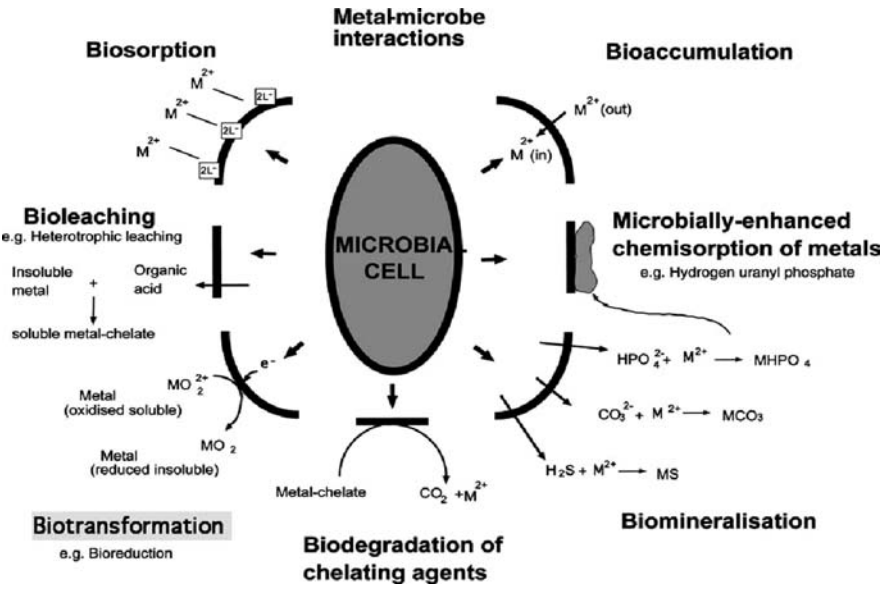

Fig. 2 Metal–microbe interactions affecting bioremediation (Tabak et al., 2005)

is through Cd-efflux ATPase. Plasmid-encoded, energy-dependent metal efflux systems involving ATPases and chemiosmotic ion/proton pumps are also reported for arsenic, chromium, and cadmium resistance in other PGPR (Roane and Pepper, 2000). The exploitation of these bacterial properties for the remediation of heavy metal-contaminated sites has been shown to be a promising bioremediation alternative (Lovley and Coates, 1997; Lloyd and Lovley, 2001). The threshold limit of metal toxicity to soil microorganisms is not conclusive, yet interaction between heavy metals and microbes do occur in nature. Microorganisms can interact with metals via many mechanisms (Fig. 2), some of which may be used as the basis of potential bioremediation strategies.

5 Bioremediation: A Natural Method for the Restoration of Contaminated Soils

Conventional approaches employed for the remediation of metals from contaminated sites are presented in Fig. 3. These methods include: (i) land filling—the excavation, transport, and deposition of contaminated soil in a permitted hazardous waste land; (ii) fixation—the chemical processing of soil to immobilize the metals,

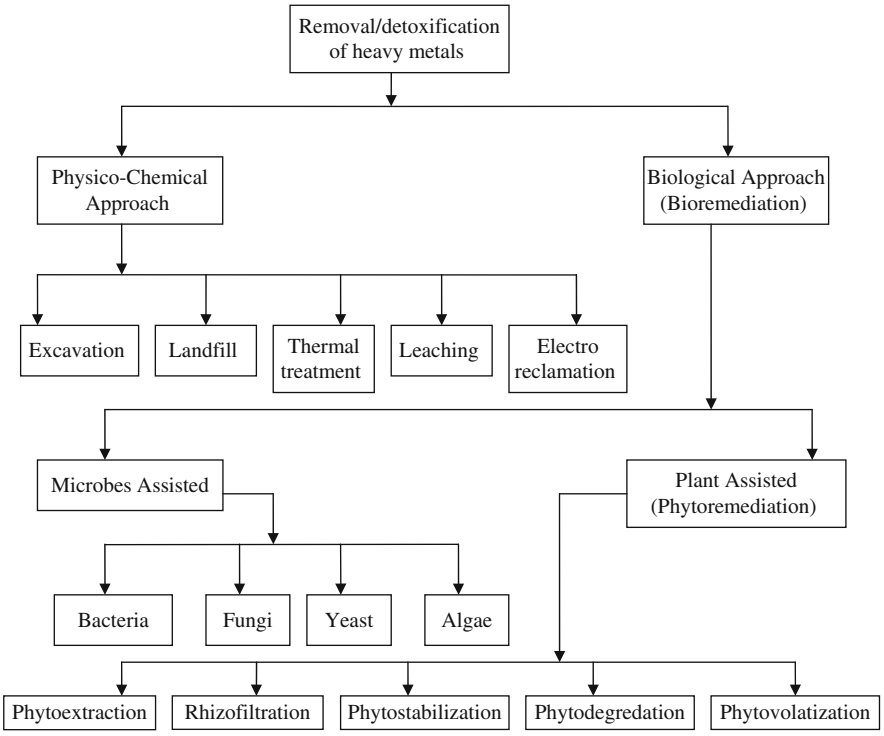

Fig. 3 Approaches employed for the remediation of heavy metals from contaminated soil

usually followed by treatment of the soil surface to eliminate penetration by water; and (iii) leaching—using acid solutions as proprietary leaching agents to distort and leach metals from soil followed by the return of clean soil residue to the site (Krishnamurthy, 2000). The applications of these processes are sometimes restricted, due to technological or economical constraints. Therefore, the search for alternative methods for restoring polluted soils in an inexpensive, less labor-intensive, safe, and environment-friendly manner is required. Such an alternative method is bioremediation, which is defined as the action of microbes or other biological systems to degrade or transform environmental pollutants under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Muller et al., 1996). Bioremediation can be applied in situ without the removal and transport of contaminated soils, and without disturbance of the soil matrix, or can be applied ex situ to soil at the site that has been removed from the site via excavation.

Therefore, managing microbial populations in the rhizosphere by using microbial inoculum consisting of a consortium of PGPR and symbiotic nitrogen fixers as allied colonizers and biofertilizers could provide plants with benefits crucial for ecosystem restoration on derelict lands (Khan, 2004). These microorganisms may be indigenous to a contaminated area (intrinsic bioremediation), or can be isolated from elsewhere and then introduced into the contaminated sites (bioaugmentation). Bioremediation depends on the functionality of organisms in the rhizosphere and the environmental conditions amenable for their growth. Advances in understanding the role of microorganisms in such processes, together with the ability to fine-tune their activities using the tools of molecular biology, has led to the development of novel or improved metal bioremediation processes.

5.1 Advantages and Limitations of Bioremediation

Bioremediation is an option that offers the possibility to degrade or render harmless various contaminants using natural biological activity. As such, it is an inexpensive, low-technology technique that generally has high public acceptance and can be consistently carried out on contaminated sites, often without affecting the fertility of soils or the metabolic activities of microbes. This remediation property helps avoid the transport of waste off-site and, consequently, the potential threats to human health and the environment that could arise during transportation. Furthermore, bioremediation can be useful for remediation of a variety of contaminants leading to their complete destruction, and when the contaminants are transformed, the toxicity of contaminants declines. Bioremediation technologies also have certain disadvantages, like the products of biodegradation may be more persistent or toxic than the parent compound. Since biological processes are often specific, they require active and specific microbial communities whose success depends on the nutrient status of soil and the levels of contaminants in the sites to be remediated. It is a time-consuming process and its laboratory successes don't easily transfer to the field environment. Since bioremediation seems to be a good

alternative to conventional clean-up technologies, research in this field is rapidly increasing. However, there is still an urgent need for molecular engineering of microbes so that they can be manipulated for better performance and wider application under diverse agroclimatic conditions.

6 Plant Growth-Promoting Rhizobacteria-Assisted Remediation of Heavy Metals

Rhizosphere soils, with a high concentration of nutrients exuded from the roots, attracts more bacteria compared to nonrhizosphere soils (Penrose and Glick, 2001). These bacteria, including PGPR, in turn facilitate the growth of the plant and this phytobacteria system has been proven to be more effective in minimizing the bioavailability and biotoxicity of heavy metals (Khan, 2005; Wani et al., 2008b). The PGPR, however, has been used largely as a growth-promoting agent in agronomic practices; substantial emphasis is being placed on them in order to exploit their bioremediation potential as well. The removal of metals from contaminated soils by PGPR can be carried out by artificial introduction of viable populations to contaminated sites (bioaugumentation), stimulation of viable native microbial population (biostimulation), biotransformation, bioreduction, bioaccumulation, and biosorption. In recent times, new metal treatments and recovery techniques based on biosorption have been explored using both dead and living microbial biomass with considerable success.

Generally, prokaryotic microbes accumulate metals by binding them as cations to the cell surface in a passive process. In this context, biosorption of metals by the PGPR strains has been studied extensively (Volesky and Holan, 1995; Lloyd and Macaskie, 2000). For example, Hernandez et al. (1998) isolated three species of bacteria belonging to the enterobacteriaceae family, which were capable of accumulating nickel and vanadium. Other potential alternative technologies currently in use involving surface complexation for metal removal include ion exchange and microprecipitation. In a study, cadmium, copper, selenium, and zinc were reported to be biosorbed by *Streptococcus faecalis, Streptococcus aureus, Bacillus subtilis, Bacillus licheniformis, Pseudomonas aeruginosa, Proteus vulgaris* and *Serratia marscecens*, in the mixtures of Gram positive and Gram negative bacteria. Generally, in Gram-positive bacteria, surface complexation occurs between organic P groups in cell-surface teichoic acid and metal contaminants. For example, uranium (VI) phosphate solids are the least soluble of all the uranium (VI) solid phases. In contrast, Gram-negative bacteria appear to have a lesser ability to sorb uranium, possibly because they lack these cell-surface organic P groups. One of the most common surface structures found both in bacteria and archaea is a crystalline proteinaceous surface layer called the S-layer, which attenuates the sorption ability of Gram-positive bacteria.

Furthermore, heavy metals in general cannot be destroyed biologically and, hence, persist in the environment. However, microorganisms can transform a wide variety of multivalent metals that pose major threats to the environment. In this regard, numerous strains of PGPR possessing metal-reducing ability have been

Table 3 Examples of Plant growth-promoting rhizobacteria (PGPR) used in bioremediation studies

Bacteria	Plant	Heavy metals	Conditions	Role of PGPR	References
Methylobacterium oryzae, Berknolderia sp.	*Lycopersicon esculentom*	Ni, Cd	Gnotobiotic and pot culture experiments		Madhaiyan et al. (2007)
Azotobacter chroococcum HKN-5	*Brassica Juncea*	Pb, Zn	Experiments in greenhouse	Stimulated plant growth	Wu et al. (2006)
Bacillus megaterium HKP-1	*Brassica Juncea*	Pb, Zn	Experiments in greenhouse	Protected plant from metal toxicity	Wu et al. (2006)
Bacillus mucillaginosus HKK-1	*Brassica Juncea*	Pb, Zn	Experiments in greenhouse	Protected plant from metal toxicity	Wu et al. (2006)
Bacillus subtilis SJ-101	*Brassica Juncea*	Ni	Experiments in growth chamber	Facilitated Ni accumulation	Zaidi et al. (2006)
Xanthomonas sp. RJ3, Azomonas sp. RJ4,Pseudomonas sp. RJ10, Bacillus sp. RJ31	*Brassica napus*	Cd	Experiment in pots	Stimulated plant growth and increased cadmium accumulation	Sheng and Xia (2006)
Pseudomonas sp, *Bacillus* sp.	Mustard	Cr (VI)	Pot experiment	Stimulated plant growth and decreased Cr (VI) content	Rajkumar et al. (2006)
Ochrobactrum, bacillus cereus	Mungbean	Cr (VI)	Experiment in pots	Lowers the toxicity of chromium to seedlings by reducing Cr (VI) to Cr (III)	Faisal and Hasnain (2006)

Table 3 (continued)

Bacteria	Plant	Heavy metals	Conditions	Role of PGPR	References
Kluyvera ascorbata SUD165 *Kluyvera ascorbata* SUD165	Indian mustard, Canola, tomato	Ni, Pb, Zn	Experiments in growth chamber	Both strains decresed some plant growth inhibition by heavy metals, No increase of metal uptake with either strain over non-inoculated plants	Burd et al. (2000)
Brevundimonas Kro13	None	Cd	Culture media	Sequestered cadmium directly from solution	Robinson et al. (2001)
Brevibacillus	*Trifolium repens*	Zn	Pot experiment	Enhanced plant growth and nutrition of plants and decreased zinc concentration in plant tissues	Vivas et al. (2006)
Variovox paradoxus, Rhodococcus sp, *Flavobacterium*	*Brassica juncea*	Cd	Experiment in Petri dishes	Stimulating root elongation	Belimov et al. (2005)
Pseudomas fluorescens	Soybean	Hg	Experiment in greenhouse	Increased plant growth	Gupta et al. (2005)
Ochrobactrum intermedium	Sunflower	Cr (VI)	Experiment in pots	Increased plant growth and decreased Cr(VI) uptake	Faisal and Hasnain (2005)
Pseudomonas sp.	Soybean, mungbean, wheat	Ni, Cd, Cr	Experiment in pots	Promotes growth of plants	Gupta et al. (2002)
Brevundimonas Kro13	None	Cd	Culture media	Sequestered cadmium directly from solution	Robinson et al. (2001)

identified (Faisal and Hasnain, 2005). As an example, among the different forms of chromium, the hexavalent form of chromium is more toxic and carcinogenic (McLean and Beveridge, 2001) due to its high solubility in water, rapid permeability through biological membranes, and subsequent interaction with intracellular macromolecules (Kamaludeen et al., 2003). Reduction of toxic hexavalent chromium to a trivalent form of chromium is considered a useful process for remediation of chromium-contaminated soil environments. The reduction/detoxification of hexavalent chromium by microbes is a low-cost process and an environmentally safe approach, and provides a viable option for protecting the soil environment from chromium toxicity. In this regard, numerous chromium-reducing PGPR—for example, *Ochrobacterium intermedium* (Faisal and Hasnain, 2005), *Pseudomonas* sp. (Rahman et al., 2007), *Bacillus* spp. (Wani et al., 2007 h) and *Mesorhizobium* sp. (Wani et al., 2008b)—have been reported.

Recently, the inoculation effects of PGPR *Methylobacterium oryzae* strain CMBM20 and *Burkholderia* sp. strain CMBM40, isolated from rice (*Oryza sativa*) tissues, on tomato (*Solanum tuberosum*), grown in nickel and cadmium-treated soil was studied (Madhaiyan et al., 2007). These bacterial strains significantly reduced the toxicity of both metals in tomatoes and promoted plant growth under gnotobiotic and pot culture conditions. It was concluded from this study that the bacterial strains reduced the uptake and consequent translocation of these metals to shoots and also synthesized phytohormones and ACC deaminase, which together accounted for increased growth of the test plant. In another study, a strain of *Pseudomonas maltophilio* transformed the mobile and toxic form of chromium (Cr VI) to a nontoxic and immobile form (Cr III), and also minimized the mobility of other toxic ions such as Hg^{2+}, Pb^{2+}, and Cd^{2+} (Blake et al., 1993; Park et al., 1999).

From these and other studies, it seems reasonable to believe that PGPR could be developed as inoculants for use in increasing plant biomass and thereby to stabilize, revegetate and remediate metal-polluted soils. Recent examples of the bioremediation of heavy metals by PGPR are shown in Table 3. Despite all these, there are certain issues that need to be addressed. These are: (i) how microorganisms induce changes in the rhizosphere and influene metal accumulation, (ii) how microbes select a particular metal for removal/detoxification from a pool of multiple metals in the contaminated sites, and (iii) how microorganisms mobilize and affect the transfer of metals to different organs of plants.

7 Rhizoremediation by Symbiotic Nitrogen-Fixing Organisms

The use of plants for rehabilitation of heavy metal-contaminated soil is an emerging area of interest because it provides an ecologically sound and safe method for restoration and remediation of polluted soils. Although numerous plant species are capable of hyperaccumulation of heavy metals, the technology is not adequate for remediating sites with multiple contaminants. A meaningful solution could be to combine the advantages of microbe-plant symbiosis within the plant rhizosphere into an effective cleanup technology. Symbiosis between plants, especially legumes and their symbionts (rhizobia), has long been studied by rhizobiologists.

The rhizosphere is an area encircling the plant root system, which is characterized by enhanced biomass productivity. The exudation of nutrients by plant roots create a nutrient-rich environment in which microbial activity is increased. Rhizosphere bacteria obtain nutrients—such as organic acids, enzymes, amino acids, and complex carbohydrates—exuded from roots. In addition, the mucigel secreted by root cells, lost root cap cells, or the decay of complete roots provides nutrients to rhizosphere microbes. In return, the bacteria convert nutrients into available forms of mineral for uptake by plants.

For example, chickpea (*Cicer arietinum*) inoculated with phosphate-solubilizing bacteria and *Mesorhizobium ciceri* were demonstrated to have increased growth, symbiosis, and yield through enhanced solubilization of phosphate and availability of sufficient quantities of N to the legume (Zaidi and Khan, 2007; Wani et al., 2007e). Furthermore, the root tips provide a steady-state redox condition and a structural surface for bacterial colonization. Researchers have exploited this symbiotic relationship for rhizoremediation technologies. The combination of bioaugmentation and phytoremediation resulting in rhizoremediation (Anderson et al., 1993) could solve some of the problems of derelict land encountered during the application of both techniques individually. In this process, the root exudates released from various plants stimulate the growth and metabolic activities of nodule bacteria that, in turn, very effectively remove the contaminats from polluted sites (Glick, 2004; Zhuang et al., 2007). Through various studies, it has conclusively been proved that the rhizobial population acquires resistance when grown in the soils contaminated heavily with metals (Pereira et al., 2006); although the information on metal tolerance by rhizobia at the molecular and cellular levels is limited. However, in recent times, the interest in rhizobia for their role in remediation of heavy metals has greatly been increased due to the fact that it influence the solubility, bioavailability, and mobility of metals in both the rhizosphere and within their legume host, and help maintain the N pool of soils and legumes.

Rhizobia grow slowly for long periods in soil, but if they infect compatible legume hosts, they can grow rapidly and successful infection by a single bacterium can lead to the formation of a nitrogen-fixing nodule on the root of legumes. Moreover, once symbiosis is established, metals may accumulate in nodules. This would be an alternative and less-expensive method of removing metals from the soil. The use of *Rhizobium* legume symbiotic interaction has therefore been suggested as a tool for rhizoremediation of metals in derelict soils (Nie et al., 2002) because the symbiotic relationship between leguminous plants and rhizobia could be exploited for the improvement of plant abilities by introducing genetically engineered rhizobia to plant roots (Sriprang et al., 2002). Recombinant rhizobia in each nodule on a root of a legume are advantageous for the expression of foreign genes that help to sequester metals in contaminated soil. For example, *Mesorhizobium huakuii* subsp. rengei strain B3 (Murooka et al., 1993; Nuswantara et al., 1999) is the N_2-fixing bacterium that establishes a symbiotic relationship with *Astragalus sinicus*—the legume that has been used as green manure in rice (*Oryza sativa*) fields in China and Japan and forms nitrogen-fixing root nodules (Chen et al., 1991). It would be advantageous if this plant could be used to increase N and, at the same time, remove metals from soil.

In other studies, the gene-encoding, metal-binding protein, tetrameric metallothionein (MTL4) (Hong et al., 2000), or arabidopsis phytochelatin synthase (PCS) (Rauser, 1995; Zenk, 1996; Cobbett, 2000) was introduced into *M. huakuii* subsp. Rengei strain B3 (Sriprang et al., 2003), which expressed under the control of a bacteroid-specific promoter, nifH or nolB (Ruvkun et al., 1982; Perret et al., 1999). Resultant recombinant strains enhanced the accumulation of cadmium in free-living cells. In another study, the most suitable plant species for rhizoremediation showed that leguminous plants such as alfalfa are suitable (Shann and Boyle, 1994). This is probably due to their ability to harbor large numbers of bacteria on their root systems. Rhizoremediation, however, depends on factors such as primary and secondary metabolites and colonization and the establishment of rhizobial, survival, and ecological interaction with other organisms in the rhizosphere.

Yet in another study, when green gram plants inoculated with metal-tolerant *Bradyrhizobium* were grown in sandy clay loam soils exposed to different levels of nickel and zinc, the bioinoculant significantly enhanced plant growth and symbiosis and reduced the uptake of nickel and zinc by plant organs (Wani et al., 2007f). Thus, the overall increase in inoculated green gram plants in metal-contaminated soils was suggested to be due to the reduction in the toxicity of metals, or by the sufficient availability of N and phytohormones synthesized by the inoculants strain to the plants. Moreover, phytohormones e.g., giberellin are reported to reduce the effect of high concentration of certain metals (e.g., cadmium) on the growth of non-inoculated soybeans (*Glycine max*) (Ghorbanli et al., 1999). In a similar study, the application of metal-tolerant rhizobia as a seed bioinoculant with reduced metal toxicity and through their PGP activities promoted the growth of lentil (*Lens esculentum*) (Wani et al., 2007 g) and chickpea (Wani et al., 2008b) in metal-treated soils. In a recent study, *Rhizobium* sp. RP5 that was tolerant to nickel and zinc was reported to protect pea (Wani et al., 2007c) plants from nickel (Fig. 4) and zinc

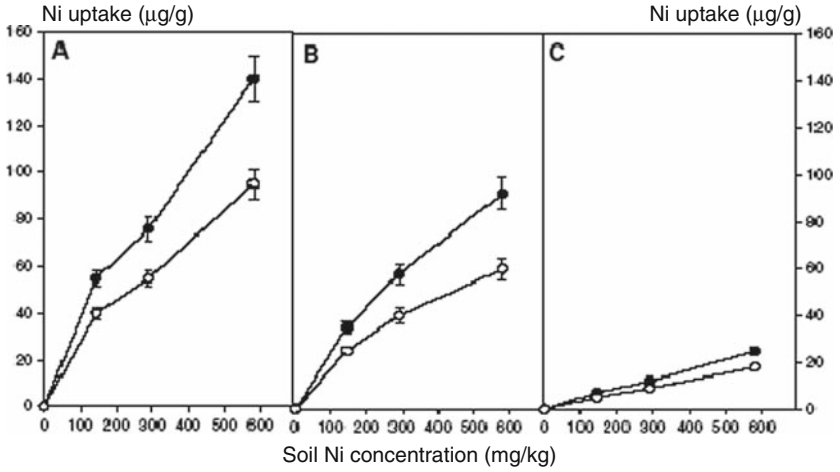

Fig. 4 Concentration of nickel in (**A**) roots, (**B**) shoots at 90 days, and (**C**) grains at 120 days after seeding pea in the absence (●) and presence (○) of bioinoculant strain RP5 with different levels of nickel. The values indicate the mean ± S.D. of three replicates. SD: standard deviation

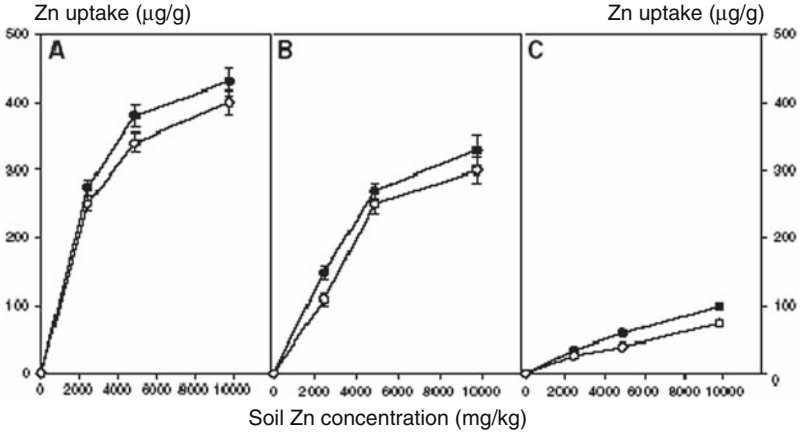

Fig. 5 Concentration of zinc in (**A**) roots, (**B**) shoots at 90 days, and (**C**) grains at 120 days after seeding pea in the absence (●) and presence (○) of bioinoculant strain RP5 with different levels of zinc. The values indicate the mean ± S.D. of three replicates

(Fig. 5) toxicity without affecting the metal uptake by roots, shoots, and grains. The plant growth-promoting effect in the presence of metals was suggested to be due to the reasons discussed earlier, in addition to their metal-reducing potential through an adsorption/desorption mechanism (Mamaril et al., 1997).

Exposure of heavy metals and other adverse environmental factors can disrupt cellular homeostasis and enhance the production of several activated species of oxygen, such as superoxide, singlet-oxygen, H_2O_2, and hydroxylradicals, which constitute an important aspect of the oxygen problem in different organs of legumes. For instance, nodules have a high capacity for producing these damaging chemicals because of the high rates of respiration, the strong reducing conditions required to reduce N_2, the tendency of leghaemoglobin to auto-oxidize, and the likely ability of nitrogenase to directly reduce oxygen (Dalton, 1995).

Moreover, plants and nitrogen-fixing rhizobia possess an efficient defense system that allows the scavenging of reactive oxygen species. One such enzyme is glutathione reductase, which detoxifies the H_2O_2 via the ascorbate-glutathione cycle (Azevedo et al., 1998). In this context, the glutathione reductase activity has been detected in the roots and nodules of inoculated pea plants grown in soils treated with nickel (Fig. 6) and zinc (Fig. 7). Generally, concentration-dependent increases of glutathione reductase activity in roots and nodules was observed for nickel and zinc in inoculated and noninoculated plants (Wani et al., 2007c). However, the glutathione activity was generally found more in the roots of both rhizobium inoculated and uninoculated plants grown in nickel- and zinc-stressed soil. This suggests that the higher concentration of these metals has probably induced the oxidative stress and generation of reactive species of oxygen, leading to the synthesis of antioxidant enzymes, which might have played a pivotal role in protecting the pea plants from the oxidative stress as also reported for other legumes (Cardoso et al., 2005; Ana et al., 2006; Lima et al., 2006). Furthermore, the enhanced growth of inoculated legumes under metal stress could also be due to the synthesis of glutathione

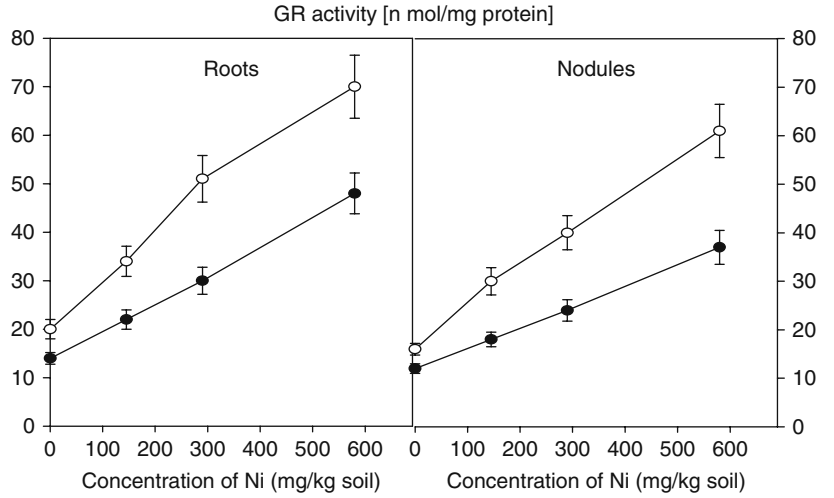

Fig. 6 Glutathione reductase (GR) activity in roots, and nodules at 90 days after seeding peas in the absence (●) and presence (○) of bioinoculant strain RP5 with different levels of nickel. The values indicate the mean ± S.D of three replicates

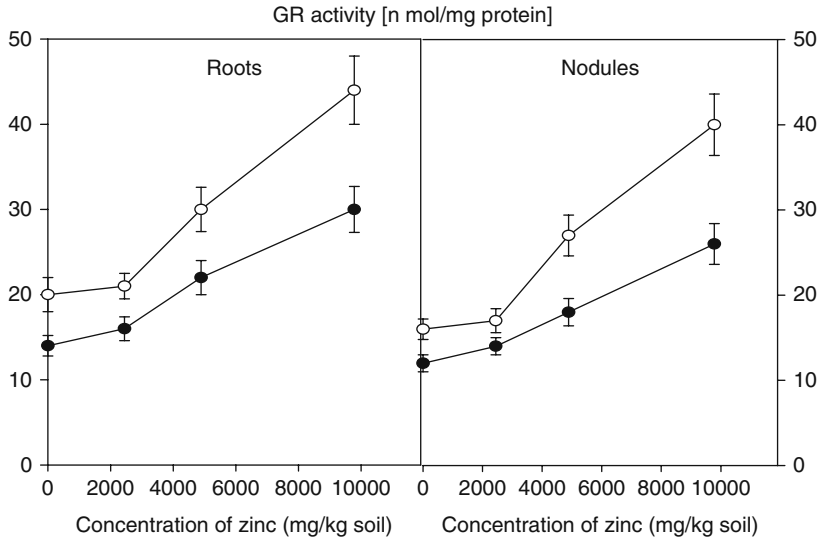

Fig. 7 Glutathione reductase (GR) activity in roots, and nodules at 90 days after seeding peas in the absence (●) and presence (○) of bioinoculant strain RP5 with different levels of zinc. The values indicate the mean ± S.D of three replicates

reductase or other detoxifying agents by rhizobia (Figueira et al., 2005; Corticeiro et al., 2006). These results could play an important role in developing biotechnological strategies for metal bioremediation procedures, and open novel prospectives for the restoration of polluted soil using legume-*Rhizobium* symbiosis.

8 Phytoremediation

Phytoremediation is an environmentally friendly and visually attractive technology that involves the use of plants to clean up pollutants from contaminated soil environments (Cunningham et al., 1995). The sensitivity or tolerance of plants towards metals is influenced greatly by plant species and genotypes. Broadly, plants can be grouped into three categories: (i) excluders, (ii) indicators, and (iii) accumulators (Fig. 8). Among these, the plants belonging to excluder groups are sensitive to metals over a wide range of soil concentrations and survive through restriction mechanisms, while indicators show poor control over metal uptake and transport processes, and correspondingly respond to metal concentrations in soils (Briat and Lebrun, 1999). Grasses (e.g., sudangrass, bromegrass, fescue, etc.) and grain and cereal crops (e.g., corn, soybean, wheat, oats, etc.) are included in the excluder and indicator groups, respectively.

Plants in the accumulator group do not prevent metals from entering the roots, and hence have evolved specific mechanisms for detoxifying high concentrations of metal accumulated in the cells. Common plants included in this group are tobacco (*Nicotiana tabacum* L.), mustard (*Brassica campestris*), and members of composite families (e.g., lettuce, spinach, etc.). Among these accumulators, there are certain plants that possess exceptionally high metal-accumulating capacity—hyper-accumulators—that allows them to survive and even thrive in heavily contaminated soils. The term hyper-accumulator was introduced for the first time by Brooks et al. (1977) to describe plants that, in their natural habitats, were capable of accumulating more than 1000 mg Ni kg^{-1} dry weight of shoots. This limit of metal accumulation is also applied to other metals (e.g., cobalt, copper, and lead), whereas for cadmium and zinc, the threshold limit is about 100 and 10,000 mg kg^{-1} shoots dry weight (Brooks, 1998; Baker et al., 2000). Most of the commonly known heavy metal accumulators belong to the brassicaceae or fabaceae families (Kumar et. al., 1995). However, more than 400 plant species have already been reported to be hyper-accumulator plants, and a considerable number of species show the capacity to

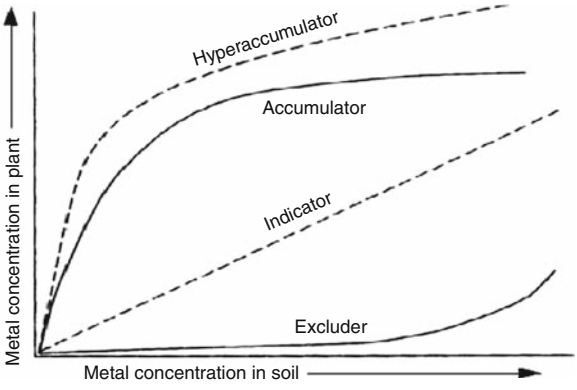

Fig. 8 Strategies of metal uptake by plants depending on the concentration of metal in soil (adapted from Adriano, 2001)

accumulate two or more elements (Zayad et al., 1998; Chaudhry et al., 1998; Hayes et al., 2003). Among the metal-accumulating plants, Indian mustard (*Brassica juncea* L. Czern) is one of the most promising species that has attracted considerable attention because of its ability to grow in heavily polluted soil together with its capacity for metal ion accumulation (Blaylock and Huang, 2000). Generally, hyper-accumulator plants accumulate one- to three-fold higher concentrations of metals than do the nonhyper-accumulator plants (Baker et al., 1994; Shen et al., 1997).

Phytoremediation technology has certain advantages and disadvantages as well. The advantages include: (i) it is a low-cost, low-energy, and environmentally friendly ecotechnology, (ii) it is far less disruptive to the soil environment, (iii) it avoids excavation and is socially acceptable, and (iv) since it involves the use of plants, it is easy to implement and maintain. The disadvantages include: (i) it is time-consuming due to the slow growth-rate of plants, (ii) it is affected by changes in agro-climatic conditions, (iii) plant biomass after remediation requires proper disposal, (iv) the contaminants may enter again into soil due to litter formation by the metal-accumulating plants, and (v) the root exudates of hyper-accumulators may enhance the solubility of pollutants and, consequently, may increase the distribution of metals into the soil environment. Thus, to make phytoremediation a viable technology, we need to search for plants that grow faster with extensive root systems, have a capacity to produce a high amount of biomass, should have lower-level contaminant uptake ability, and be able to accumulate higher amounts of contaminants or engineer common plants with hyper-accumulating genes.

8.1 How Plants Help Restore Degraded Soil

Roots are the first organ of plants that come in contact with heavy metals in contaminated soils and, after uptake by the roots, metals are translocated to different organs of plants (Fig. 9). In soils heavily contaminated with metals, plants suffer various injuries leading to the death of the plants (Rout and Das, 2003; Wani et al., 2006) by inactivation of photosynthesis (Wani et al., 2007b), synthesis of proteins and DNA(Asada, 1994), stomatal action, and generation of free radicals (Breen and Murphy, 1995) as presented in Fig. 10. However, in order to survive in the metal-polluted soils, plants could accumulate, sequester, or synthesize metal-binding complexes (phytochelatins)—a simple γ–glutamyl peptide (Grill et al., 1985) —whose formation in response to the challenge of heavy metals is one of the few truly adaptive stress responses observed in plants.

Generally, plants adopt one or a combination of the following mechanisms to protect itself from metal toxicity. Such processes could be: (i) phytoextraction—this is a low-cost technique through which metal is removed or concentrated into plant parts, producing a mass of plants and contaminants (usually metals) that can be transported for disposal or recycling; (ii) phytodegradation or rhizodegradation—metals are degraded by the proteins or enzymes produced by plants and their associated microbes; or (iii) rhizofiltration—metal is absorbed by plant roots; (iv) phytostabilization—in this technique, metals are immobilized and the mobility and

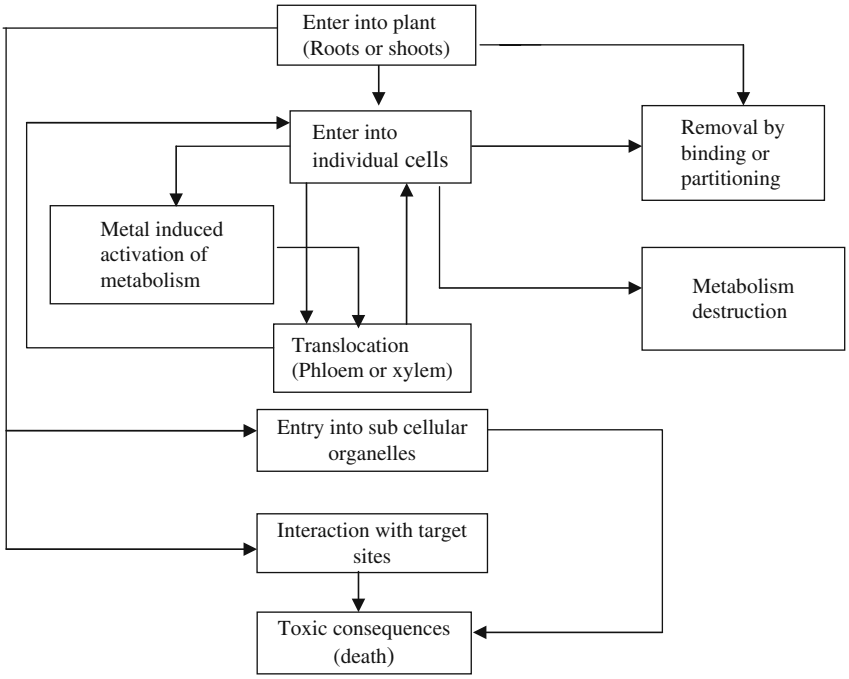
Enter into plant (Roots or shoots)
Enter into individual cells
Removal by binding or partitioning
Metal induced activation of metabolism
Metabolism destruction
Translocation (Phloem or xylem)
Entry into sub cellular organelles
Interaction with target sites
Toxic consequences (death)

Fig. 9 A flow chart showing the sequence of events from metal entry into a plant to the death of the plant

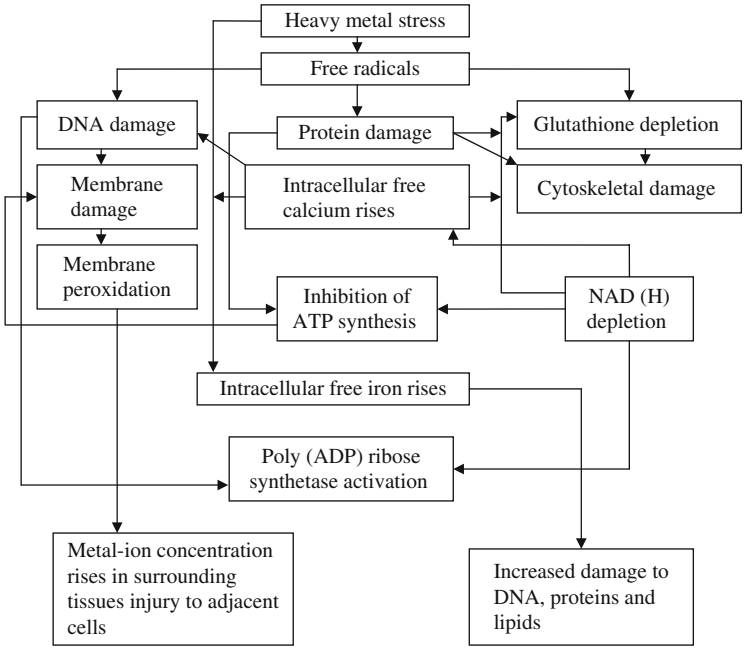

Fig. 10 Metabolism of plants damaged by heavy metals

bioavailability of metals to plant roots are reduced. Leachable constituents are adsorbed and form a stable complex with the plant structure from which the contaminants will not re-enter the environment. Another process is phytovolatization—volatization of pollutants by plants from the soil into the atmosphere. Phytoremediation techniques are generally applied to large field sites where the costs of other remediation methods are high or not practical.

8.2 Plant Growth-Promoting Rhizobacteria Affecting Phytoremediation

Contaminated soils are often nutrient poor or sometimes nutrient deficient due to the loss of beneficial microbes. However, such soils can be made nutrient rich by applying metal-tolerant microbes, especially the PGPR, which would provide not only the essential nutrients to the plants growing in the contaminated sites but would also play a major role in detoxifying heavy metals (Mayak et al., 2004) and thus helping plants capable of remediating heavy metals (Glick, 2003). For example, when applied to soils amended with nickel, zinc, lead, and chromate PGPR *Kluyvera ascorbata* SUD165, isolated from metal-contaminated wetlands near Sudbury in Ontario, Canada, have shown to increase the growth of canola (*Brasica rapa*) while protecting the plants from nickel toxicity (Burd et al., 1998). Similarly, nickel resistant *Kluyvera ascorbata* protected tomatoes (*Lycopersicon esculentum* L.), Indian mustard (*Brassica campestris*), and canola plants when grown in soils supplemented with nickel lead and zinc (Burd et al., 2000). Moreover, the growth-promoting rhizobacteria *Variovorax paradoxus*, *Rhodococcus* sp., and *Flavobacterium* sp. stimulated root elongation of Indian mustard seedlings either in the presence or absence of toxic cadmium (Belimov et al., 2005), suggesting that these bacterial strains could be developed as inoculants to improve the growth of the metal-accumulating Indian mustard in the presence of toxic cadmium concentrations, and for the development of plant inoculant systems useful for phytoremediation of polluted soils. Similarly, the canola plants inoculated with *Enterobacter cloacae*, when grown in the presence of arsenates, grew to a significantly greater extent than nontransformed canola plants (Nie et al., 2002).

In yet other studies, *Ochrobacterium intermedium* and *Bacillus cereus* protected green gram plants against chromium toxicity (Faisal and Hasnain, 2006), while inoculation of *Ochrobacterium intermedium* improved the overall growth of sunflower (*Helianthus annus*) when grown in metal-amended soils (Faisal and Hasnain, 2005). In other studies, metal-tolerant, growth-promoting rhizobacteria has also shown a substantial protection for plants against metal toxicity, and consequently improved the growth, symbiosis, and seed yield of plants (Chaudri et al., 2000; Wani et al., 2007c, 2008b). The increase in the growth of agronomically important crops grown in metal-stressed soils by applying metal-tolerant rhizobacteria was attributed to the ability of rhizobacterial strains to mitigate the toxic effects of metals using mechanisms as discussed earlier in addition to providing plants with sufficient amounts of growth-promoting substances. It may therefore be advisable for growers to

inoculate plants with such rhizobacterial microbes to increase plant biomass and thereby stabilize, revegetate and restore/remediate heavy metal-polluted soils.

9 Conclusion

Remediation of metal-polluted soils using biological systems (both microbes and plants) is an emerging area of interest and has shown substantial progress in situ that needs to be further consolidated through field trials in different agroclimatic zones of the world. Understanding the mechanistic basis of the physical, chemical, and biological rhizosphere processes, and the interactions between hyper-accumulators and nonaccumulators and PGPR will be important in better modeling the full impact of phytoremediation in the restoration of derelict lands. Furthermore, the remediation of heavy metal-contaminated sites using rhizobacteria is an exciting area of research, since these organisms can easily and inexpensively be mass-produced. Therefore, the molecular engineering of both microbes and plants with desired genes would help immensely in enhancing the efficiency of growth promoting-rhizobacteria mediated or plant-based remediation of contaminated soils. However, to make bioremediation a successful option for the remediation of contaminated soils, some of the problems needs to be critically addressed. Such problems are: (i) why PGPR fail to perform in comparatively extreme environments, (ii) how rhizobacteria colonize plant roots and interact selectively with other indigenous microflora, (iii) how the remediation effects will change under field conditions, (iv) research is needed to investigate various aspects of metal accumulation by plant organs, and (iv) we also need to understand the mechanisms involved in mobilization and transfer of metals to develop further strategies and optimize the phytoremediation process. These are some of the challenges that need the urgent attention of scientists before the potential of bioremediation in remediating the metal polluted soils can be appreciated.

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Phosphates for Pb Immobilization in Soils: A Review

Patricia Miretzky and Alicia Fernandez Cirelli

Abstract In its soluble ionic forms, lead (Pb) is a toxic element occurring in water and soil mainly as the result of human activities. The bioavailability of lead ions can be decreased by complexation with various materials to decrease their toxicity. Pb chemical immobilization using phosphate addition is a widely accepted technique for immobilizing Pb from aqueous solutions and contaminated soils. The application of different P amendments causes Pb in soils to shift from forms with high availability to the most strongly bound Pb fractions. The increase of Pb in the residual or insoluble fraction results from formation of pyromorphite $Pb_5(PO_4)_3X$, where X = F, Cl, Br, OH, and the most stable environmental Pb compounds under a wide range of pH and Eh natural conditions. Accidental pyromorphite ingestion does not yield bioavailable lead, because pyromorphite is insoluble in the intestinal tract. Numerous natural and synthetic phosphate materials have been used to immobilze Pb: apatite and hydroxyapatite, biological apatite, rock phosphate, soluble phosphate fertilizers such as monoammonium phosphate (MAP), diammonium phosphate (DAP), phosphoric acid, biosolids rich in P, phosphatic clay, and mixtures. The identification of pyromorphite in phosphate-amended soils has been carried out by different nondestructive techniques such as x-ray diffraction (XRD), scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX), x-ray absorption fine structure (XSFS), transmission electron microscopy (TEM), and electron microprobe analysis (EMPA). The effectiveness of in situ Pb immobilization has also been evaluated by sequential extraction (SE), by the toxicity leaching procedure (TCLP), and by a physiologically-based extraction procedure simulating metal ingestion and gastrointestinal bioavailability to humans (PBET). Efficient Pb immobilization using P amendments requires increasing the solubility of the phosphate phase, and of the Pb species phase, by inducing acid conditions. Although phosphorus addition seems to be highly effective, excess P in soil and its potential effect on the eutrophication of surface water, and the possibility of

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arsenic enhanced leaching remains a concern. The use of mixed treatments may be a useful strategy to improve their effectiveness in reducing lead phyto- and bioavailability.

Keywords Lead remediation · Soils · Phosphorous materials · Pyromorphite precipitation

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1 Introduction

Lead reaches the soil environment through pedogenic means (related to the origin and nature of the parent material) and anthropogenic processes. Anthropogenic activities—primarily associated with industrial processes, manufacturing, and the disposal of domestic and industrial waste materials—are the major source of lead contamination of soils (Adriano, 2001). It is not possible to consider the presence of lead and its compounds in the environment, and its potential toxicity to the ecosystem and to the human population, without considering its chemical speciation and mineral form. Knowing lead speciation is vital; not only to predicting its mobility and bioavailability, but also in the assessment of risk to living organisms because insoluble forms of lead cannot easily be absorbed by biota.

A critical variable in the risk from soil Pb is the bioavailability of the Pb. The form of Pb in soil directly influences its bioavailability, as do soil properties. For humans, most concern stems from ingested Pb. This commonly occurs when Pb-containing soil particles are consumed. The Pb must be released as Pb^{2+} in the digestive fluids before it can be absorbed into the body. A review on in vivo studies dealing with Pb ingestion can be seen in Hettiarachchi and Pierzynski (2004).

Lead can be carried in water—either dissolved or as waterborne particles. However, few compounds of lead dissolve readily in water, although most of this lead is then precipitated as a solid and becomes incorporated in the sediments at the base of the watercourse or ocean. In most cases, lead in the soil is relatively insoluble and

has low mobility. Thus, soils contaminated with lead retain high-lead contents for many hundreds, even thousands, of years. The half life of lead in soil has been estimated as 740–5,900 years (Alloway and Ayres, 1997). Lead compounds are more mobile under acidic conditions, which can occur in mine wastes or from landfill leachate.

The presence of Pb in soils is affected by three principal processes: (1) precipitation as a slightly soluble mineral phase; (2) adsorption on the clay fraction, the Fe and Mn oxides, the alkaline earth carbonates and silicate lattices; and (3) formation of relatively stable complexes by interaction with soil organic matter (Davis, 1995; Bradl, 2004). The mobility of lead depends upon many factors: Pb speciation and total Pb soil content, the type of soil, soil pH, moisture content of the soil, and water infiltration from rainfall or other drainage. In soils, Pb shows very low aqueous phase concentrations (1 to $< 0.01\%$ of total Pb) with Pb^{2+} being the principal dissolved specie (Sauvé et al., 2000).

Solubility is generally used in reference to the stability of a given Pb mineral; nevertheless, solubility is a thermodynamic parameter, only defined when the system reaches equilibrium. Mechanisms of retention and release of soil contaminants are, in most cases, not instantaneous equilibria but rather time-dependant processes. In as dynamic a system as soil, the mineral dissolution kinetics must be considered. Slow rates of Pb dissolution from different minerals results in decreased Pb bioavailability and a minor risk of soil Pb movement into groundwater (Laperche et al., 1996). Thus, in addition to solubility, the dissolution rate must be taken into account when evaluating the stability of a Pb compound. Pb phosphates have low solubility—they are several orders of magnitude less soluble than the analogous carbonates and sulphates (Nriagu, 1984).

Remediation of contaminated soils and the disposal of metal-contaminated soils is a very expensive and arduous task. As an alternative, in situ chemical immobilization is less expensive than excavation and land-filling, and provides a long-term remediation solution through the formation of stable metal minerals and/or precipitates (Vangronsveld and Cunningham, 1998). The decrease in metal solubility (and therefore in mobility) reduces the risk of heavy-metal transport from contaminated soils to groundwater and surface water. According to the USEPA, remediation action is considered when the total soil Pb content exceeds $400\,mg.kg^{-1}$ (USEPA, 1996).

Pb chemical immobilization using phosphate addition is a widely accepted technique to immobilize Pb from aqueous solutions and in contaminated soils (Ma et al., 1993, 1994a,b, 1995; Ryan et al., 2001) to reduce Pb plant uptake (Cotter-Howells and Caporn, 1996; Laperche et al., 1997; Hettiarachchi et al., 2000), to mitigate acid mine drainage (Melamed et al., 2003), and to minimize leachable Pb in industrial wastes (Eighmy et al., 1997, 1998; Crannell et al., 2000). Phosphate treatment has been proposed as a "Best Management Practice" for firing ranges where P occurs in its metallic forms and several other phases (carbonates, oxides) (Chrysochoou et al., 2007).

There are several possible Pb-phosphate minerals that can be formed in P-amended soils (Lindsay, 1979; Traina and Laperche, 1999; Essington et al., 2004; Porter et al., 2004). In acidic soils Pb phosphates predominate over Pb oxides and

carbonates, which form in alkaline soils (Lindsay, 1979; Davis, 1995). Numerous studies demonstrated that the members of the pyromorphite family $Pb_5(PO_4)_3X$, where X = F, Cl, Br, OH, are the most stable environmental Pb compounds under a wide range of pH and Eh natural conditions. The presence of As in many contaminated soils must be taken into consideration, as competition between phosphate and arsenate for adsorption sites may cause As mobilization (Peryea and Kammereck, 1997; Boisson et al., 1999).

Different P sources with different solubilities may impact the effectiveness of Pb immobilization. Numerous natural and synthetic phosphate materials have been used—apatite and hydroxyapatite, rock phosphate, soluble phosphate fertilizers such as monoammonium phosphate (MAP), diammonium phosphate (DAP), phosphoric acid, etc. (Ma et al., 1993; Ruby et al., 1994; Ma et al., 1995; Cotter Howells and Caporn, 1996; Basta et al., 2001; McGowen et al., 2001; Ryan et al., 2001; Yang et al., 2001; Mavropoulos et al., 2002; Caoet al., 2004; Hettiarachchi and Pierzynski, 2004). We provide a review of the literature concerning the Pb immobilization in soil by phosphate natural and synthetic materials with a focus on the role of complexation and chemical speciation in the geochemical process.

2 Pb Geochemistry in P-Amended Soils

Application of different P amendments caused Pb in soils to shift from forms with high availability—exchangeable, carbonate, Fe-Mn oxide, organic-matter bound—to the most strongly bound Pb fractions—sulphide or residual (Beti and Cunningham, 1997; Ma and Rao, 1997; Ryan et al., 2001; Cao et al., 2002, 2003; Chen et al., 2003; Knox et al., 2003; Melamed et al., 2003; Tang et al., 2004; Chen et al., 2007). The increase of Pb in the residual fraction results from formation of pyromorphite.

Cao et al. (2002) and Melamed et al. (2003) reported a decrease in the carbonate-bound Pb soil fraction up to 40%, and 10% of the Fe − Mn oxide bound fraction, while the residual fraction increased up to 60%. The more effective treatments should convert greater amounts of Pb from the nonresidual to the residual fraction. It has been reported that some of the Pb transformation from the nonresidual to residual forms occurred during the extraction process (Ryan et al., 2001; Schekel et al., 2003), however the fact that the residual Pb soil fraction increases with time strongly demonstrated that conversion of soil Pb to more stable forms actually occurs in the field (Cao et al., 2002; Scheckel and Ryan, 2002). This redistribution of Pb resulted in less phytotoxiciy, as indicated by greater plant growth and lower metal concentration in plant tissue (Laperche et al., 1997; Boisson et al., 1999; Zhu et al., 2004; Chen et al., 2007; Deydier et al., 2007).

The interaction of Pb and P through the formation of pyromorphite is an important buffer mechanism controlling the migration and fixation of Pb in water, soils, and wastes, reducing Pb solubility as well as bioavailability (Bolan et al., 2003). Phosphorus amendment could be an effective means of immobilizing lead in drinking or sewage, since accidental pyromorphite ingestion does not yield bioavailable

lead (Arnich et al., 2003). Pyromorphite is highly stable even in low pH, and will not dissolve appreciably in the human digestive system (Cheng and Hseu, 2002).

The solubility products of pyromorphites are extremely low, $10^{-71.6}$, $10^{-76.8}$, $10^{-78.1}$ and $10^{-84.4}$ for fluoro-, hydroxyl-, bromo-, and chloropryromorphites, respectively (Chen et al., 1997b). Chloropyromorphyte is several orders of magnitude less soluble than hydroxyl-, bromo- and fluoropyromorphytes, and due to the ubiquity of chloride in nature, chloropyromorphite is the dominant form of pyromorphites (Ryan et al., 2001). The chemical and physical attributes of chloropyromorphite (low entropy state with aging) suggested that its persistence would endure most environmental conditions, thus making Pb immobilization via phosphorus an ideal remediation mechanism (Schekel and Ryan, 2002).

Because pyromorphites are the most stable Pb phosphate minerals in natural environmental conditions, thermodynamics predict that other solid phases would be converted to pyromorphite by a dissolution-precipitation process. Experimental evidence shows that various Pb compounds such as cerrusite, anglesite, galena, and also lead in contaminated soils are partly transformed to chloropyromorphite after reaction with hydroxyapatite, with the rate-limiting step being the dissolution/oxidation of Pb in the bearing solid. (Laperche et al., 1996; Zhang et al., 1998 ; Zhang and Ryan, 1999a, b; Ryan et al., 2001; Yang and Mosby, 2006). Nevertheless, efficient Pb immobilization using P amendments requires enhanced solubility of metals by inducing acidic conditions (Cao et al., 2003).

As mentioned before, thermodynamic considerations show that of the several lead phosphate minerals and salts, chloropyromorphite is the most stable in a soil environments. To convert all other forms of lead into this mineral, it is necessary to change all the calcium in the soil into hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Since calcium is always 10-20 times more abundant than phosphate in soil, very large amounts of phosphate need to be added to increase the amount of phosphorus in the soil up to $400\,\mu molg^{-1}$(PO_4/Ca ratio $= 3/5$) (Porter et al., 2004). This large input of P ($> 4\%$ relative to soil Pb concentration) could be a concern for nearby water environments, and also could alter the soil structure, as virtually all the calcium in soil will be precipitated as apatite, decreasing the soil friability (Scheckel and Ryan, 2004).

The identification of pyromorphite in phosphate-amended soils has been carried out by different nondestructive techniques (Table 1) such as x-ray diffraction (XRD) (Ma et al., 1993; 1994a, b; Zhang and Ryan 1999b; Mavropoulos et al., 2002), SEM coupled with energy dispersive x-ray spectroscopy (SEM-EDX) (Laperche et al., 1997; Chen et al., 1997b; Zhang and Ryan, 1999a; Ryan et al., 2001; Arnich et al., 2003), x-ray absorption fine structure (XSFS) (Cotter-Howells et al., 1994; Ryan et al., 2001), transmission electron microscopy (TEM) (Zhang et al., 1998; Zhang and Ryan, 1999a; Mavropoulos et al., 2004; Srinivasan et al., 2006), and electron microprobe analysis (EMPA) (Yang et al., 2001).

The effectiveness of in situ Pb immobilization has also been evaluated by sequential extraction (SE) (Berti and Cunningham, 1997; Ryan et al., 2001; Chen et al., 2007), by the toxicity leaching procedure (TCLP) where leaching concentration is compared against the USEPA limit of 5 mg L^{-1}(Hettiarachchi et al., 2000; Cao et al.,

2001; Wilson et al., 2006), and also by a physiologically based extraction procedure simulating metal ingestion and gastrointestinal bioavailability for humans (Ruby et al., 1996; Basta et al., 2001). Schekel et al. (2003) reported that during the extraction steps of SE, and because the kinetics of pyromorphite precipitation are fast, the solubilized P from the amended soils reacted with Pb released by the extractant to form pyromorphite-type minerals in a secondary precipitation. Thus, the resulting residual fraction Pb values were greater than those from spectroscopic studies. Scheckel et al. (2005) discussed the limitations of these methods and concluded that appropriate application of advanced, molecular-level spectroscopic methods provide more conclusive and accurate results than the use of sequential extraction analysis.

3 Different Sources of P for Pb Immobilization in Soils

3.1 Amendments with Apatite

Apatite $Ca_{10}(PO_4)_6(OH, F, Cl)_2$ is a promising candidate for heavy-metal stabilization because its crystal structure is tolerant to many ionic substitutions and the complete replacement of Ca^{2+} by Ba^{2+}, Sr^{2+}, Cd^{2+} and Pb^{2+} and P^{5+} by V^{5+}, Cr^{5+} and As^{5+} (Srinivasan et al., 2006).

Most of the studies on Pb immobilization by apatite (Table 1) were carried out with Pb^{2+} aqueous solutions or Pb from contaminated soils and solid apatite (Ma et al., 1993, 1994a, b; Xu and Schwartz, 1994; Laperche et al., 1996, 1997; Chen et al., 1997a, b; Zhang and Ryan, 1998, 1999a, b, Zhang et al., 1998; Ryan et al., 2001; Seaman et al., 2001; Mavropoulos et al., 2002, 2004; Knox et al., 2003; Srinivasan et al., 2006), although previously dissolved hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ was also used (Manecki et al., 2000; Arnich et al., 2003). Synthetic hydroxyapatite was used by Ma et al. (1993, 1994a, b); Xu et al. (1994); Laperche et al. (1996); Boisson et al. (1999) ; Mavropoulus et al. (2002, 2004) and natural apatite by Chen et al. (1997a, b); Laperche et al. (1997); Knox et al. (2003).

Application of hydroxyapatite to aqueous Pb or Pb contaminated soils at a P/Pb molar ratio of 3/5 (the same molar ratio as in chloropyromorphite) has been suggested (Ma et al., 1993; Laperche et al., 1996), but higher ratios have been used for soluble P (Zhang and Ryan, 1998; Basta et al., 2001; Hettierachchi et al., 2001). When hydroxyapatite is added to soil, mainly as a source of P, it also has a liming value in addition to supplying Ca. The liming action of apatite is due to the presence of free $CaCO_3$ as an impurity, and to the fact that it consumes H^+ in the dissolution process, thereby reducing soil acidity (Knox et al., 2003).

Two different main mechanisms have been proposed for the immobilization of lead by hydroxyapatite:

1) The first mechanism involves hydroxyapatite dissolution, followed by phosphate reaction with dissolved Pb and precipitation of pure hydroxypyromorphite (Ma et al., 1993; Xu and Schwartz, 1994; Chen et al., 1997b; Zhang and Ryan 1998, 1999a, b; Zhang et al., 1998; Lower et al., 1998).

The chemical equations involved are:

$$Ca_{10}(PO_4)_6(OH)_2(s) + 14H^+ = 10Ca^{2+} + 6H_2PO_4^- + 2H_2O \quad \log K = 28.92 \text{ (HA dissolution)} \tag{1}$$

$$10Pb^{2+} + 6H_2PO_4^- + 2H_2O = Pb_{10}(PO_4)_6(OH)_2(s) + 14H^+ \quad \log K = 8.28 \text{ (pyromorphite precipitation)} \tag{2}$$

Several authors have reported that the overall reaction is fast, hydroxyapatite-reduced initial aqueous Pb concentration from $100\,mgL^{-1}$ to $< 1\mu gL^{-1}$ in less than 10 min (Ma et al., 1993; Xu and Schwartz, 1994). Because the reaction rate is controlled by hydroxyapatite dissolution and availability of soluble P, lowering soil pH was found to significantly enhance the dissolution of soil Pb and favor the reaction towards pyromorphite formation (Laperche et al., 1996; Zhang et al., 1998). Although from a thermodynamic approach pyromorphite has the potential to control Pb solubility, pyromorphite formation is kinetically controlled by pH, the solubility of the phosphate source, and the solubility of the Pb species (Chrysochoou et al., 2007).

The composition of the solution influences the interaction of dissolved Pb with apatites. Ma et al. (1994a) reported that hydroxyapatite was transformed in hydroxypyromorphite in the presence of NO_3^-, Cl^-, F^-, SO_4^{2-}, and CO_3^{2-}. The presence of other metal ions also influences the reaction of dissolved Pb with hydroxyapatite, specially when the metal/ Pb ratio > 7 (M = Zn, Cd, Ni , Cu, Fe (II), Al) (Ma et al., 1994b).

Microscopic and spectroscopic studies showed that pyromorphite can precipitate either homogeneously from solution or heterogeneously on the surface of apatites that serve as a substrate for nucleation and as source of phosphate ions (Manecki et al., 2000). Evidence of homogeneous nucleation in the precipitation of hydroxypyromorphite was shown by Lower et al. (1998) using atomic in situ and ex situ force microscopy (AFM) techniques. Diffusion of phosphate away from dissolving hydroxyapatite was the rate-limiting step.

The evidence for this hydroxyapatite dissolution and pyromorphite precipitation mechanism was based on x-ray diffraction (XRD) and scanning electron microscopy (SEM) studies performed on the solid phase (Table 1). Also, analysis of solution pH and Ca^{2+} and $PO4^{3-}$ concentrations during Pb^{2+} immobilization was performed. The second mechanism involves an ion exchange between Pb^{2+} in solution and Ca^{2+} on hydroxyapatite lattice (Suzuki et al., 1981; Takeuchi and Arai, 1990; Shashkova et al., 1999).

The chemical equation involved is:

$$Ca_{10}(PO_4)_6(OH)_2(s) + xPb^{2+} = Ca_{(10-x)}Pb \times (PO_4)_6(OH)_2(s) + xCa^{2+}$$

This mechanism could lead to the formation of mixed apatites by adsorption of Pb or by dissolution of hydroxyapatite followed by coprecipitation (Laperche and Traina, 1998). However, using spectroscopic techniques and synthetic

hydroxyapatite, these researchers showed that even at low Pb^{2+} concentration, the reaction product was pyromorphite and not (Pb − Ca) apatite. The principal mechanism involved was dissolution of apatite followed by precipitation of pyromorphite, rather than adsorption of Pb into apatite particles. Arnich et al. (2003) reported that the mechanism involved in the immobilization of lead as pyromorphite, identified by XRD, was an ion exchange between Ca^{2+} ions in hydroxyapatite lattice and Pb^{2+} ions in solution.

Mavropoulos et al. (2002) demonstrated that the reaction of Pb with hydroxyapatite was controlled by hydroxyapatite (HA) dissolution and the formation of a new lead and calcium solid solution—$Pb_{(10-x)}Ca \times (PO_4)_6(OH)_2$(PbCaHA)—that transforms into hydroxypyromorphite with time, with Pb^{2+} ions occupying Ca^{2+} sites. The existence of PbCaHA as an intermediate phase was confirmed by XRD and TEM (Mavropoulos et al., 2004).

The capability of three-dimensionally ordered macroporous hydroxyapatite (3DOM) to immobilize Pb was studied by Srinivasan et al. (2006). The macroporous product consisted primarily of hydroxyapatite (>80%) together with amorphous calcium phosphate. By using XRD and SEM, the researchers showed that Pb adsorption resulted in the total destruction of the macroporous structure of hydroxyapatite and the accumulation of pyromorphite (30%) and lead carbonate (9%) among the disrupted network. Because the hydroxyapatite content (61%) was high after exposure to the Pb solution, the authors suggested that the amorphous apatite be dissolved preferentially to release phosphate ions that rapidly precipitate pyromorphite, promoting further dissolution of the phosphate. The direct ion exchange process on the surface of hydroxyapatite was a slower process, overwhelmed by the reactions mentioned before.

The theoretical studies on Pb immobilization by hydroxyapatite showed that two mechanisms—pyromorphite precipitation and Pb adsorption on hydroxyapatite lattice—could be contributing mechanisms. The influence of each of them is strongly dependent on pH and pore solution chemistry (Chrysochoou et al., 2007).

3.2 Amendments with Water-soluble Phosphates

The dissolution-precipitation mechanism mentioned in the previous section suggests that phosphates and hydrogen phosphates that can dissolve more easily than hydroxyapatite may be employed as Pb amendments (Table 2). Soluble phosphate sources could provide an abundance of solution P and increase the efficiency of metal-phosphate mineral formation (McGowen et al., 2001). Water-soluble phosphates include potassium mono and dihydrogen phosphate (KH_2PO_4, K_2HPO_4); calcium monohydrogen ($CaHPO_4$) and calcium dihydrogen phosphate $Ca(H_2PO_4)_2$; phosphoric acid (H_3PO_4) and the phosphates used as fast-release fertilizers—single super phosphate (SSP) and triple superphosphate (TSP), monoammonium phosphate ($NH_4H_2PO_4$) (MAP) and diammonium phosphate ($(NH_4)_2HPO_4$) (DAP), with calcium monohydrogen phosphate ($CaHPO_4$) being the principal component

of super phosphates (Bolan et al., 2003). Diammonium phosphate is a major source of P fertilizer and represents approximately 70% of the total U.S. production (McGowen et al., 2001). The use of sodium phosphate is not recommended because it causes later problems in soils.

Application of hydroxyapatite or phosphate rocks to calcareous soils could restrict the precipitation of Pb as pyromorphite or pyromorphite-like minerals because dissolution of the hydroxyapatite or phosphate rocks is limited. Application of H_3PO_4 that would lower soil pH and provide highly soluble P constitutes an effective remedial treatment in calcareous Pb-contaminated smelter soils by the formation of pyromorphite (Yang et al., 2001).

$$5Pb^{2+} + 3H_2PO_4^- + Cl^- = Pb_5(PO_4)_3Cl_{(s)} + 6H^+ \log K = -25.5 \qquad (3)$$

Neutralization of protons generated during pyromorphite precipitation showed in Eq. 3 favors the reaction toward the pyromorphite formation, thus initially acidifying soil followed by gradually increasing soil pH would enhance the transformation of Pb.

Statistical analysis by linear combination fitting (LCF) applied to XAFS spectroscopic data were utilized by Scheckel and Ryan (2004) to obtain in situ evidence of principal Pb species in amended soils with H_3PO_4. They found that the addition of H_3PO_4 promoted pyromorphite formation, and that the rate of formation increased with increasing P concentration (up to 45%). Similar results were found by the above investigators using triple superphosphates enriched with Fe.

Yang and Mosby (2006) tested different methods of application of H_3PO_4 in Pb-contaminated soils and reported that roto-tilling was the most effective in terms of the soluble P homogeneity and the reduction of Pb bioaccessibility in the treated soil zone. Strawn et al. (2007) also studied H_3PO_4 amendments on Pb-contaminated soils by means of XRD and EMPA techniques, and concluded that that after addition of P, distinct mineralogical changes occurred in soils, such as oxidation of siderite. In the original soil, Pb was associated with poorly crystalline Fe and Mn oxides, and the added P resulted in a primary association with Fe oxide phases. They also observed that soil pore waters were undersaturated with respect to chloropyromorphite and plumbogummite $PbAl_3(PO_4)_2(OH)_5H_2O$, in contrast with control soil, indicating that P resulted in diminished Pb solubility.

Phosphoric acid was regarded as the most effective amendment (USEPA, 2001) because of its easy delivery and superior ability to dissolve Pb^{2+}-form existing minerals and transform it into pyromorphites. The amendment dosage proposed by USEPA (2001) is 3% PO_4 by weight for soils.

Other different sources of soluble P in Pb immobilization in contaminated soils were tested (Cao et al., 2002, 2003; Melamed et al., 2003; Sugiyama et al., 2003; Schwab et al., 2006). A pilot scale field experiment was conducted by Cao et al. (2002, 2003) in a Pb-contaminated site using three treatments (P/Pb molar ratio of 4.0): (a) 100% P from H_3PO_4; (b) 50% P from H_3PO_4 + 50% from $Ca(H_2PO_4)_2$; and (c) 50% P from H_3PO_4 + 50% from phosphate rock. The role of H_3PO_4 in the mixture was to solubilize the Pb-bearing minerals and also the phosphate rocks,

increasing the readily available Pb in the soil. They concluded that although all the treatments immobilized Pb due to the formation in situ of insoluble pyromorphite-like minerals, the H_3PO_4 and phosphate rock treatment produced less impact on soil pH reduction and less leaching of soluble P—a potential eutrophication risk. Similar results were reported by Chen et al. (2003) and Melamed et al. (2003).

Pb immobilization in aqueous solutions using calcium phosphate and calcium hydrogen phosphates proceeded through the P dissolution and pyromorphite precipitation mechanism (Sugiyama et al., 2003). Soluble phosphate as diammonium phosphate at a rate of $2{,}300\text{mg P kg}^{-1}$ provided effective immobilization of Pb in smelter-contaminated soil (McGowen et al., 2001), however the contaminated soil had received limestone application previous to the amendment. Application of liming materials, together with diammonium phosphate, may be necessary to reduce potential soil acidification in nonalkaline soils. Berti and Cunningham (1997), immobilized Pb in soils in situ by using potassium dihydrogen phosphate. Phosphate added as low as 0.5% P was able to reduce leachable Pb to below the regulatory limit of 5 mg L^{-1} in soils with a Pb content of $1{,}200-3{,}500\text{Pb mg kg}^{-1}$. The authors mentioned before also reported promising results in the use of the physiologically based extraction test (PBET) that determines the soil's Pb bioaccessibility—a chemical surrogate measurement for soil Pb bioavailability in animals after soil ingestion.

The choice of amendment with soluble phosphate salts to remediate Pb-contaminated soils must be carefully studied, not only because of the material cost but for the secondary contaminant problems. Due to the high solubility of these phosphates, and to the fact that phosphate is a nutrient, this technique can lead to eutrophication of groundwater and surface waters (Cotton-Howells and Caporn, 1996). It has been reported that P adsorption decreases with increasing additions of phosphorous (Heredia and Fernández-Cirelli, 2007)

3.3 Amendments with Phosphate Rock

We have already mentioned that amendments with highly soluble P may increase the risk of P-induced eutrophication; therefore, the use of igneous or sedimentary phosphate rock, primarily fluoroapatite (FA) $Ca_{10}(PO_4)_5F_2$, to immobilize aqueous Pb or Pb from contaminated soils may reduce that risk. The structure of fluoroapatite is similar to that of hydroxyapatite, F^- occupying the OH^- sites on the six-fold axis. However, hydroxyapatite is much more soluble than fluoroapatite ($\log K_{ps} = -55.9$ and -110.2, respectively) (Table 2). In general, the solubility of phosphate rocks increases with an increase in carbonate substitution (Ma et al., 1995). The following equations correspond to the dissolution of an apatite with carbonate substitution followed by precipitation of carbonated fluoro pyromorphite-like minerals.

$$\begin{aligned} Ca_{10}(PO_4)_3(CO_3)_3FOH(s) + 6H^+ = 10Ca^{2+} + 3H_2PO_4^- + 3CO_3^{2-} \\ + F^- + OH^- \text{ (dissolution)} \end{aligned} \tag{4}$$

$$10Pb^{2+} + 3H_2PO_4^- + 3CO_3^{2-} + F^- + OH^- = Pb_{10}(PO_4)_3(CO_3)_3FOH(s) + 6H^+ \text{ (precipitation)} \quad (5)$$

The removal of Pb from aqueous solutions using natural phosphate rock was investigated by Mouflih et al. (2006), reporting that the principal mechanism at low pH was dissolution of natural phosphate and precipitation of chloropyromorphite. The addition of phosphate rock to convert the soil Pb to the more stable pyromorphite was accomplished by several authors (Laperche et al., 1997; Ma and Rao, 1999; Hettiarachchi et al., 2000; Basta et al., 2001; Cao et al., 2002; Lin et al., 2005). Ma et al. (1995) reported a reduction in dissolved Pb of 22−100%, after addition of phosphate rocks to 13 contaminated soils.

The main mechanism of Pb immobilization is the dissolution of the phosphate rock followed by the precipitation of fluoropyromorphite $Pb_{10}(PO_4)_6F_2$ (Chen et al., 1997a,b; Cao et al., 2003, 2004). The formation of fluoropyromorphite $Pb_{10}(PO_4)_6F_2$ has been confirmed by XRD and SEM (Cao et al., 2004).

$$Ca_{10}(PO_4)_6F_2 + 12H^+ = 10Ca^{2+} + 6H_2PO_4^- + 2F^- \text{ (dissolution)} \quad (5)$$

$$10Pb^{2+} + 6H_2PO_4^- + 2F^- = Pb_{10}(PO_4)_6F_2 + 12H^+ \text{ (precipitation)} \quad (6)$$

It has been suggested that Pb has a higher probability of being incorporated into the apatite structure by isomorphic substitution because of Pb^{2+} ionic radius = 0.133 nm being greater than $Ca^2 +$ radius = 0.094 nm. The isomorphic substitution for those metals with lower radius, Cu^{2+} (0.069 nm) or Zn^{2+} (0.074 nm), would be less favorable (Boisson et al., 1999).

As seen in Eqs. 5 and 6, although fluoroapatite dissolution consumes H^+, the same amount of H^+ is liberated in the fluoropyromorphite precipitation, so there should not be a change in the solution pH (Mavropoulos et al., 2002). Cao et al. (2004) reported that up to 78.3% of adsorbed Pb was irreversibly chemisorbed onto the phosphate rock via fluoropyromorphite precipitation, but they observed a reduction in pH and concluded that another surface mechanism besides phosphate rock dissolution and fluoropyromorphite precipitation may have contributed to the pH decrease. They proposed that complexation occurred on the phosphate rock surface sites, partially displacing H^+ ions as shown in the following equation (PR: phosphate rock)

$$PR - OH + Pb^{2+} = PR - OPb^+ + H^+ \quad (7)$$

Unlike soluble P fertilizers, such as single superphosphate, triple superphosphate, and diammonium phosphate, phosphate rocks neutralize soil acidity during the dissolution process and present a liming effect. Also, phosphate rocks usually contain some free calcium carbonate ($CaCO_3$) that is also a liming agent per se.

It has been shown that the formation of pyromorphites decreases when using phosphate rocks of limited dissolution in alkaline soils (Laperche et al., 1997; Zhang et al., 1997). Soil acidic conditions play an important role in Pb immobilization using P amendments, because in neutral or alkaline soils, the solubility of Pb and P minerals is low. Melamed et al. (2000)reported that when phosphate rock is used to immobilize Pb at pH 8.7, soluble P concentration is low, resulting in a relatively low soil Pb immobilization. However, at pH 3.7, phosphate rock dissolves and Pb immobilization is instantaneous, forming a pyromorphite-type material.

Garrido et al. (2006) found that after soil amendment with phosphate rocks, most of the Pb was associated to the Fe and Al oxyhydroxide fraction, and that this distribution did not change during incubation for one year. Mixing both H_3PO_4 and phosphate rock with contaminated soil, and applying PR as a layer in the soil column were effective in reducing Pb migration (73−79%), minimizing soil acidification and P eutrophication (Yoon et al., 2007).

A study of the grain size of phosphate rock and the effectiveness of Pb immobilization was performed by Chen et al. (2006). The results showed that phosphate rock of the smallest grain size (<35 μm) was superior to that with large grain size for in situ remediation technology. However, effectiveness of solid phosphate is hindered by the size of the particles. In fact, even fine-ground solid phosphate particles are not mobile in soils, a fact which prevents solid phosphate from being delivered to the contaminated zone and reacting with Pb^{2+} adsorbed in soils (Liu and Zhao, 2007).

3.4 Amendments with Other Phosphorus Materials

The application of biosolids may constitute an alternative method for Pb immobilization, due to the fact that biosolids often have high concentrations of Fe and P and $>$ 50% organic matter (Brown et al., 2003). High P biosolid compost has been used to reduce the bioavailability and bioaccessibility of urban soil Pb (Brown et al., 2004). Also, Farfel et al. (2005) reported that soils with $>$ 800 mg Pb kg^{-1} were successfully amended with 110−180 dry t /ha of biosolids compost-rich in Fe and P.

The mechanisms responsible for the reduced Pb availability when using biosolids are not clear, although it is assumed that adsorption and posterior precipitation could be the principal ones (Li et al., 2000). Because biosolids contain high concentrations of Fe and Mn combined with the high organic-matter content, ferrihydrite formation is favored. In the presence of Pb, ferrihydrite forms surface inner sphere complexes that, with time, lead to the precipitation of a more crystalline Pb-bearing Fe oxide (Scheinost et al., 2001).

Bone meal, hydroxyapatite as the principal mineral constituent, has been identified as a potential source of P due to the moderate solubility associated with its carbonate content and poorly crystalline structure. Hodson et al. (2001) and Sneddon et al. (2006) reported a significant decrease in solution Pb when bone meal

was added to soil, although the Pb immobilization mechanism could not be determined. Due to the bovine spongiform encephalopathy crisis, bone meal production can no longer be used to feed cattle and must be safely disposed and transformed. The commercial cost of bone meal is 25% that of the synthetic hydroxyapatite (Hodson et al., 2001), thus bone meal could be a cost-effective natural phosphate source for P amendment.

The ashes produced as meat and bone meal combustion residue, a calcium-phosphate rich material, were use by Deydier et al. (2007) for in situ remediation of Pb-contaminated solutions and soils. These researchers determined by use of chemical kinetics and XRD analysis that Pb was immobilized as pyromorphite and Pb carbonate dehydrate ($PbCO_3.2H_2O$).

Fishbone, a natural apatite-rich substance (70% apatite equivalent), was used to immobilize Pb^{2+} from aqueous solutions to below detectable levels (Admassu and Breese, 1999). The effectiveness of phosphatic clay, a by-product of the phosphate mining industry, for immobilizing Pb^{2+} from aqueous solutions was studied by Singh et al. (2001), with precipitation of fluoropyromorphite being the principal immobilization mechanism. The results were confirmed by XRD and SEM.

In recent years, environmental application of nanoscale zero-valent iron has attracted considerable interest, because of improved reactivity, especially when it is stabilized by cellulose to prevent agglomeration. Liu and Zhao (2007) tested a new class of cellulose-stabilized iron phosphate $Fe_3(PO_4)_2.8H_2O$ (vivianite) nanoparticles for immobilizing Pb^{2+} in three representative soils (calcareous, neutral, and acidic), and/or solid and hazardous wastes. The significant decrease of Pb^{2+} availability in the soils was attributed to the formation of pyromorphite minerals through the iron phosphate nanoparticle amendment. Under acidic conditions, the following reactions follow:

$$Fe_3(PO_4)_2.8H_2O + 4H^+ = 3Fe^{2+} + 2H_2PO_4^- + 8H_2O \ \log K = 6.8 \quad (8)$$

$$5Pb^{2+} + 3H_2PO_4{}^- + Cl^- = Pb_5(PO_4)_3.Cl(s) + 6H^+ \ \log K = 12.6 \quad (9)$$

At $pH > 7.2$, equations become

$$Fe_3(PO_4)_2.8H_2O + 2H^+ = 3Fe^{2+} + 2HPO_4^{2-} + 8H_2O \ \log K = -7.6 \quad (10)$$

$$5Pb^{2+} + 3HPO_4^{2-} + Cl^- = Pb_5(PO_4)_3.Cl(s) + 3H^+ \ \log K = 34.2 \quad (11)$$

The use of vivianite-stabilized nanoparticles in Pb-contaminated soil converted a large fraction of water soluble/exchangeable and carbonate-bound Pb^{2+} to highly stable pyromorphite minerals, resulting in enhanced Pb^{2+} immobilization. The stabilized nanoparticles produced much less phosphate leachate in comparison to phosphate salts, reducing the risk of eutrophication.

3.5 Mixed Phosphate Amendments

Different P amendments on Pb-contaminated soils were tested by Hettierachchi et al. (2001)—triple superphosphate, phosphate rock, acetic acid and triple superphosphate, and H_3PO_4 in different doses. A significant reduction of bioavailable Pb was determined by PBET, resulting in phosphate rock—the most effective in the stomach phase—and triple superphosphate in the intestinal phase. Similar results were reported by Cao et al. (2003), resulting in a mixture of H_3PO_4 and phosphate rock—the most efficient in immobilizing Pb with less impact on soil pH and less leaching of soluble P.

Zwonitzer et al. (2003) studied the effect of soluble P (KH_2PO4) and PR in the immobilization of Pb, Cd, and Zn from contaminated soil, resulting in a soluble source more effective for decreasing Pb bioavailability. No conclusive results were found for Cd^{2+} and Zn^{2+}.

An international interlaboratory study was performed to test the ability of different P amendments—phosphate rock, triple superphosphate, and H_3PO_4—to reduce the availability of Pb in situ (Brown et al., 2005). P, added as either triple superphosphate or H_3PO_4, was the most effective as was shown by increased plant growth, reduced metal concentration in plant tissue, reduced soil solution and extractable Pb, and reduced bioavailability of soil Pb.

The effectiveness of the addition of different P amendments (hydroxyapatite, phosphate rock, and single super phosphate) in the bioaccessibility of Pb in soils to humans, using in vitro tests was studied by Tang et al. (2004). These investigators found that single superphosphate had the best performance in minimizing the Pb bioaccessibility in the gastric phase, and the hydroxyapatite in the small intestinal phase.

Zhu et al. (2004) also studied the effect of different P amendments (hydroxyapatite, phosphate rock, single superphosphate, and the combination hydroxyapatite and single superphosphate) in a Pb-polluted alkaline soil. The Pb bioavailability was determined by plant uptake and sequential extractions. Hydroxyapatite was effective in transforming Pb from nonresidual fractions to residual form, reducing the Pb bioavailabitiy in soil and also the Pb accumulation in vegetable crops. The low effectiveness of phosphate rock was due to soil alkalinity.

Chen et al. (2007) evaluated the efficiency of different phosphorus amendments: natural hydroxyapatite, phosphate rock, triple superphosphate, and diammonium phosphate in contaminated soils to reduce the bioavailability of lead, concluding that phosphate rock and hydroxyapatite efficiently decreased the uptake of lead by plants due to the formation of pyromorphite in soils and roots. Although diammonium phosphate was efficient to immobilizing Pb, it decreased soil pH, causing leaching of heavy metals from soil. The immobilization and bioavailability of Pb was determined by plant uptake, SEM-EDX and SE.

A mix treatment—the formation of pyromorphite by amendment of contaminated Pb soil with soluble phosphate Na_2HPO_4, and by the biochemical action of the roots of *Agrostis capillaris* proposed by Cotter-Howells and Caporn (1996)—turned out to be more ecologically acceptable than the addition of large amounts of soluble phosphate. Also, the effects of combining two microbial and three different apatite amendments on the bioavailability of lead in a shooting-range soil were studied by Wilson et al. (2006).

4 Conclusion

The main goal of in situ soil-remediation techniques is to reduce mobility, bioavailability, and toxicity of the metal contaminant, although total metal concentration is not significantly reduced by amendment addition. The results of these studies demonstrate that pyromorphite formation can be accomplished by the reaction of Pb in a contaminated soil and different phosphorus sources, increasing the geochemical stability of soil Pb. The redistribution of lead from more chemically labile forms to residual phases resulted in less bioavailability and less phytotoxicity, indicated by greater plant growth and lower metal concentrations in the plant tissue (Knox et al., 2003; Brown et al., 2004; Ryan et al., 2001). The chemical stability of chloropyromorphite under different environmental conditions (Scheckel and Ryan, 2002) makes Pb immobilization by phosphorous amendments a very effective remediation technique, since accidental pyromorphite ingestion does not yield bioavailable lead, being pyromorphite insoluble in the intestinal tract (Zhang et al., 1998; Arnich et al., 2003).

We must take into consideration that the formation of pyromorphite as a solubility controlling phase is kinetically controlled by pH, the solubility of the phosphate phase, and the solubility of the Pb species, so soluble or acidic phosphate sources are necessary for in situ successful treatment (Chrysochoou et al., 2007). The use of lime to restore soil pH reduces pyromorphite formation.

Phosphoric acid was regarded as the most effective amendment (USEPA, 2001) because of its easy delivery and superior ability to dissolve Pb^{2+} from existing minerals and transform it in pyromorphites. Although phosphorus addition seems to be highly effective, excess P in soil and its potential effect on the eutrophication of surface water remains a concern. The use of mixed treatments could improve their effectiveness in reducing lead phyto and bioavailability, however inconsistencies in measurements make it difficult to assess their effectiveness (Brown et al., 2004, 2005).

The efficiency of the P amendment in Pb-contaminated soil depends on the type of soil and the nature and extent of the contamination. The type and rate of the P source and also the application management to be used in the soil amendment, should be carefully studied. Also, the risk of primary P leaching and eutrophication of surface water sources, and the possibility of As enhanced leaching, are of great concern.

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Cadmium Phytotoxicity: Responses, Mechanisms and Mitigation Strategies: A Review

Abdul Wahid, Muhammad Arshad and Muhammad Farooq

Abstract Contamination of soils with cadmium is a critical factor affecting soil properties and plant growth. Cadmium is toxic to most plants in trace amounts, while other plants show varying tendencies to grow under relatively high cadmium levels. Some plants can bind the absorbed cadmium to their cell walls. Roots, being directly exposed, always accumulate greater amounts of cadmium than shoots. Effects of cadmium toxicity on above-ground parts include plant stunting, leaf rolling, chlorosis and necrosis, diminished stomatal conductance and gas exchange, perturbed leaf water and nutrient status, hormonal imbalance, production of oxidative stress, and enhanced peroxidation of membrane lipids. Plants use various mechanisms to cope with cadmium, which include synthesis of metal chelating proteins, expression of enzymatic and nonenzymatic antioxidants, organic acids, and plant root–mycorrhizal association. Cadmium toxicity can be alleviated by the exogenous use of metal chelators, and organic and inorganic sources. Finding strategies to bind cadmium in soil systems and better understanding of species diversity for cadmium tolerance, cadmium-responsive genes, and the molecular basis of cadmium-tolerance may be important strategies for coping with this ever-increasing problem.

Keywords Cadmium · Phytoavailability · Oxidative stress · Chelation · Genotypic variability · Nutrients

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1 Introduction

Heavy-metal pollution is an ever-increasing worldwide issue (Kashem and Singh, 1999; Yagdi et al., 2000; Jamali et al., 2007). Heavy metals, including cadmium, mercury, copper, and zinc, accumulate in soils and plants in variable quantities and create a range of agricultural and human health-related issues. Major soil pollutants and their sources include pesticide and fertilizer use, solid waste and sludge disposal, and processes including electroplating, batteries, welding, smelting, and pigments (Lugon-Moulin et al., 2006). In many countries, wastewater is used for irrigation, which is a main cause of the accumulation of even essential micronutrients to phytotoxic levels in soils. Thus, to lower the damaging effects of toxic metals on plants, a suitable pretreatment of wastewater and soils prior to its use is imperative (Ghafoor et al., 1997; Ye et al., 2000).

Cadmium is an important toxicant in affecting plant productivity (Prasad, 1995; Thiebeauld et al., 2005; Wahid and Ghani, 2008) and has a long biological half-life (Himly et al., 1985). Uninterrupted application of industrial wastewaters for irrigation over the past few decades has led to the accumulation of heavy metals in upper soil strata, which is not only phytotoxic (Helal et al., 1998) but also reduces the bioavailability of essential metals (Ghafoor, 2000). Cadmium is a nonessential element and is released into the environment from various industries like power stations, heating systems, metal industries, and urban traffic. Its wide use in industries constitutes an important source (Sanita di Toppi and Gabbrielli, 1999). It never occurs in isolation in natural environments, rather it is often found in association with lead and zinc as a guest metal. Therefore, greater accumulation of cadmium in plants appears due to its accompaniment of other metals (Baker et a1., 1994). A nonpolluted soil contains 0.04–0.32 μM cadmium, while its concentration in moderately polluted soil solutions varies from 0.32 μM to ∼1 mM (Wagner, 1993). In water, however, cadmium concentrations vary from none to about 0.01 μg/L (Anonymous, 2006).

Cadmium toxicity in plants is observed at the whole plant, as well as at cellular and molecular levels; the most important of which includes perturbation of

metabolic pathways such as photosynthesis, energy transduction, protein synthesis, and nutritional disorders. Plants entail certain adaptive mechanisms to cope with these adverse effects, including the synthesis of metal binding and chelating proteins, antioxidants, and osmoprotectants. Furthermore, the adverse effects of cadmium can be alleviated by employing breeding and selection strategies, functional genomics approaches, exogenous use of organic and inorganic chemicals, and membrane stabilizers. This review emphasizes important effects, mechanisms, and some mitigation strategies employed to overcome the adverse impacts of cadmium on plants.

2 Cadmium in the Soil System

Physico-chemical properties of soil and soil solution greatly affect the phytoavailability of cadmium (Cao et al., 2007). However, data pertaining to the effects of added cadmium on the properties of soil are scarce. This is most likely due to the fact that cadmium is toxic to plants in very low amounts and plants can not thrive in heavily cadmium-contaminated soils, which may alter their physiological properties. The available literature shows that soil properties, including chemical form and speciation, valence state, solubility characteristics, interactions with essential metals, presence of cadmium-chalators, ascorbate, metallothionein, and cadmium-complex formation are important (Mengel et al., 2001). Stimulation of oxygen consumption on soil incubation with 0.01 and 10 mg cadmium per kg soil has been shown to uncouple respiratory phosphorylation (Naidu and Reddy, 1988). It has been shown that growing crops on heavy-metal dredged sediments can modify their physico-chemical state by lowering the pH, enhancing redox potential by mechanical action of root, production of soluble organic compounds, and promoting microbial activity. These processes enhance the mobility and bioavailability of already existing heavy metals, which may be a great threat to upcoming vegetation as well as the environment (Marseille et al., 2000).

At higher cadmium levels, a substantial accumulation of nitrite nitrogen (NO_2–N) suggests that cadmium is toxic to soil nitrification (Rother et al., 1982). Ammonium nitrogen (NH_4^+–N) lowers the soil pH upon dissociation of nitrogen from proton (H^+) and enhances the bioavailability of cadmium (Lorenz, 1994; Mengel et al., 2001). Other studies show that the combined effects of a range of soil properties, particularly cation exchange capacity (CEC), organic matter, and pH, control the concentration of cadmium in solution, and its sorption and desorption in soil (Gray et al., 1999). Of these, soil pH is more important; acidic pH enhances—while alkaline pH lowers—its phytoavailability (Guo et al., 2007). By contrast, chelating agents in the organic matter may chelate cadmium and allow the soil microflora to flourish (Jones et al., 1987). In addition to chelating agents, the addition of phosphate as KH_2PO_4 increases the soil pH, negative charge, and adsorption by the soil, and enhances soluble and exchangeable cadmium-fraction, resulting in its poor phytoavailability (Bolan et al., 2003).

3 Cadmium Phytotoxicity Responses

Cadmium does not have any beneficial physiological role in plants, but when accumulated, it affects all aspects of growth and development (Fig. 1). The most frequently observed effects of cadmium-phytotoxicity are inhibition of root elongation, perturbation of water relations, suppression of photosynthetic activity, a decline in biomass production, and even the plant death. It is strongly phytotoxic and produces a vast array of changes at morpho-anatomical, physiological, and biochemical levels. These effects are strongly dependent upon the stage of plant growth, the level of cadmium applied, and the physico-chemical nature of the plant growth medium (Table 1).

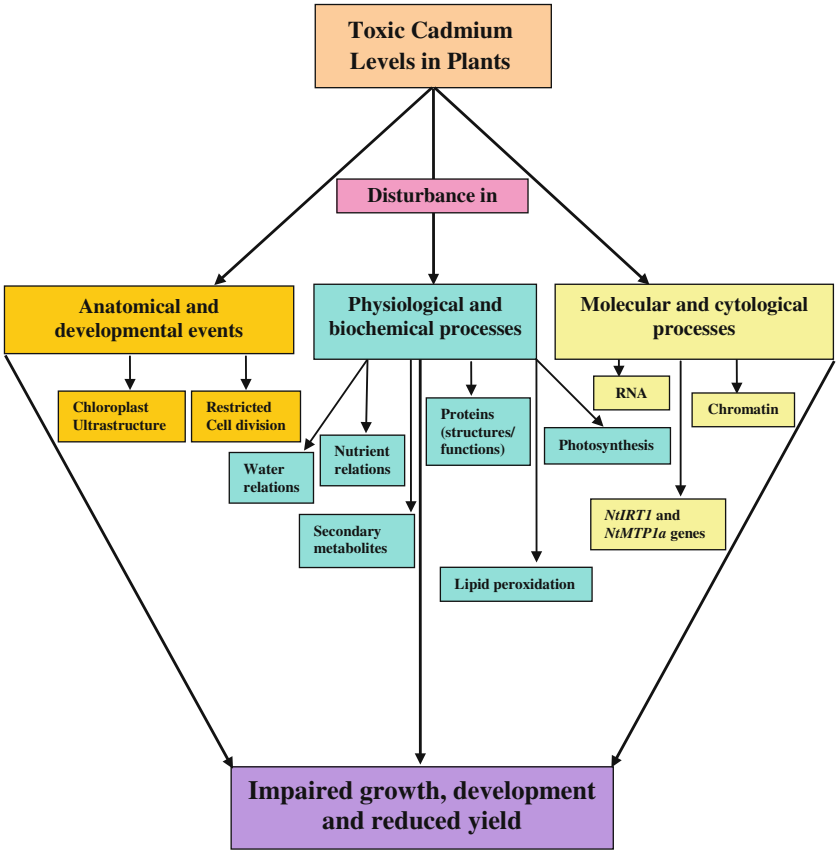

Fig. 1 Proposed mechanisms of damage by toxic levels of cadmium in plants. The cadmium adversely affects the plant growth and development and these effects are evident on the physiological, biochemical, and developmental phenomena of plants

Table 1 Phytotoxic concentrations of cadmium in plant species and their growth stages. The phytotoxic levels of cadmium vary greatly with plant species and cadmium accumulation in various plant parts is highly linked to the levels of cadmium in the growth medium

Plant species	Plant part analyzed	Growth medium	Maximum cadmium concentration (on dry weight basis)	Reference
Cape gooseberry (*Physalis peruviana*)	Leaf	Nutrient solution (200 mg/L cadmium; pH 5.5)	~3.0 mg/kg	Thiebeuld et al. (2005)
As above	Root	As above	~70 mg/kg	As above
Pepino (*Solanum muricatum*)	Leaf	As above	~35.0 mg/kg	As above
	Root	As above	~120 mg/kg	As above
Pepper (*Capsicum annuum*)	Leaf	As above	~33.0 mg/kg	As above
	Root	As above	~110 mg/kg	As above
Tobacco (*Nicotiana tabacum*)	Leaf	As above	~31.0 mg/kg	As above
	Root	As above	~75 mg/kg	As above
Tomato (*Lycopersicon esculentum*)	Leaf	As above	~15.0 mg/kg	As above
	Root	As above	~82 mg/kg	As above
Tomato	Shoot	Nutrient solution (50 μM $CdCl_2$)	~65 μmol/g	Mediouni et al. (2006)
	Root	As above	~25 μmol/g	As above
Greengram (*Vigna* sp.)	Root	Soil	~2.2 μg/g	Wani et al. (2007)
	Shoot	As above	~0.8 μg/g	As above
	Grain	As above	~0.5 μg/g	As above
Spinach (*Spinacea oleracea*)	Shoot	Cd-polluted soil	2.81 μg/g	Helal et al. (1998)
	Root	As above	12.10 μg/g	As above
Willows (*Salix* sp.) clones	Leaves	Cd-contaminated soil (Cambisol)	47–89 mg/kg	Tlustos et al. (2007)
As above	Twigs	As above	18–40 mg/kg	As above

Table 1 (continued)

Plant species	Plant part analyzed	Growth medium	Maximum cadmium concentration (on dry weight basis)	Reference
As above	Leaves	Cd-contaminated soil (Chernozem)	0.93–3.66 mg/kg	As above
As above	Twigs	-do-	0.91–2.18 mg/kg	As above
As above	Leaves	Cd-contaminated soil (Fluvisol)	9.51–101 mg/kg	As above
As above	Twigs		6.24–53.2 mg/kg	As above
Maize (*Zea mays*)	Seedling shoot	Black soil	~42 mg/kg	Cao et al. (2007)
As above	Seedling root	As above	~140 mg/kg	As above
Soybean (*Glycine max*)	Seedling shoot	As above	~70 mg/kg	As above
As above	Seedling root	As above	~155 mg/kg	As above
Chamomile (*Matricaria chamomilla*)	Shoot	Nutrient solution	~550 μg/g	Kovacik et al. (2006)
As above	Root	As above	~4500 μg/g	As above
Almond (*Prunus dulcis*)	Leaves	Nutrient solution	~75 μg/g	Nada et al. (2007)
As above	Root	As above	~3200 μg/g	As above
Barley (*Hordeum vulgare*)	Glum	Nutrient solution	~7 mg/kg	Chen et al. (2007b)
As above	Awn	As above	~21 mg/kg	As above
As above	Rahis	As above	~13 mg/kg	As above
As above	Stem	As above	~39 mg/kg	As above
As above	Grain	As above	~14 mg/kg	As above
As above (cv. Hamidiye)	Shoot	As above	138 mg/kg	Tiryakioglu et al. (2006)

Table 1 (continued)

Plant species	Plant part analyzed	Growth medium	Maximum cadmium concentration (on dry weight basis)	Reference
As above	Root	As above	3743 mg/kg	As above
As above (cv. Tokak)	Shoot	As above	110 mg/kg	As above
As above	Root	As above	3164 mg/kg	As above
Phyllanthus amarus	Aerial parts	Loam soil (pH 7.7)	85 ppm	Rai et al. (2005)
As above	Root	As above	65 ppm	As above
Wheat (*Triticum aestivum*) cv. PBW343	Leaf	Soil	~60 μg/g	Khan et al. (2007)
As above	Root	As above	~330 μg/g	As above
As above cv. WH542	Leaf	As above	~90 μg/g	As above
As above	Root	As above	~580 μg/g	As above
Sunflower (*Helianthus annuus*)	Young leaf	Nutrient solution	42.43 μg/g	Di Cagno et al. (1999)
As above	Mature leaf	As above	37.06 μg/g	As above
As above	Stem	As above	25.02 μg/g	As above
As above	Root	As above	240.82 μg/g	As above
Durum wheat (*Triticum turgidum* var *Durum*); a low Cd line	Shoot	As above	~0.6 μg/g	Hart et al. (2006)
As above	Root	As above	~9.0 μg/g	As above
As above; a high Cd line	Shoot	As above	~1.1 μg/g	As above
As above	Root	As above	~6.0 μg/g	As above
Arabidopsis halleri	Shoot	As above	~3000 mg/kg	Zhao et al. (2006)
As above	Root	As above	13000 mg/kg	As above

3.1 Morphology, Growth and Yield Responses

Cadmium is one of the most toxic and mobile metallic elements in soil (Pinto et al., 2004). It is a potential contaminant of the soil and environment. Its salts are highly water-soluble and cadmium in ionic form is highly mobile within the phloem and, therefore, easily translocated to various plant parts (Mengel et al., 2001; Pinto et al., 2004). A high mobility of cadmium in the phloem is due to its properties, such as a high affinity and binding with the sulphydryl group of amino acids and some proteins in the sieve tube (Reid et al., 2003). It diminishes the elongation of both shoot and root, and dry matter production by plants, primarily due to hampered photosynthetic activity (Azevedo et al., 2005b). Plant species and even genotypes differ in their ability to absorb, transport, and accumulate cadmium in a number of species, including cotton (Wu et al., 2004), pea (Metwally et al., 2005), lupin (Brennan and Mann, 2005), salix (Tlustos et al., 2007), mungbean (Wahid and Ghani, 2008), *Avena strigosa* and *Crotalaria juncea* (Uraguchi et al., 2006), and many other plant species (Table 1).

The symptoms visible on plant parts directly indicate the intensity of prevailing stress. Visual cadmium-toxicity symptoms on plants are leaf rolling, chlorosis of leaf and stem, leaf necrosis, tip-burning, plant-stunting, browning of roots and yellowing of leaves (Baryla et al., 2001; Mishra et al., 2006; Ghani and Wahid, 2007; Wahid et al., 2007b), impaired growth, and decline in yield due to higher cadmium tissue concentrations (Schützendübel and Polle, 2001; Dhir et al., 2004; Wu et al., 2006). Such effects appear due to iron and phosphorus deficiencies or reduced manganese transport (Godbold and Hüttermann, 1985; Alcantara et al., 1994). Uptake of cadmium from the substrate and its accumulation in the plant is quite fast, and toxicity symptoms are discernible within 48 h of exposure (Tiryakioglu et al., 2006). Plant stunting due to cadmium-stress occurs mainly due to diminished water uptake (Costa and Morel, 1993; Perfus-Barbeoch et al., 2002) and reduced leaf gas exchange. (Chugh and Sawhney, 1999). These effects can be of immense help in the diagnosis of stress effects and in adopting appropriate strategies to increase stress tolerance and, ultimately, selection of promising germ plasm (Wu et al., 2006; Wahid and Ghani, 2008).

Species and cultivars display marked differences for cadmium accumulation and tolerance (Table 1). These differences are evident from physiological processes like decline in production of reactive oxygen species and enhanced antioxidative defense (Tiryakioglu et al., 2006). Inter- and intraspecific variations in cadmium concentration have been found in certain crops at various growth stages (Florijin and Van Beusichem, 1993; Li et al., 1995; Metwally et al., 2005). For example, comparison of two *Nicotiana* species revealed that *Nicotiana rustica* was more tolerant of cadmium than *Nicotiana tabacum* due to a difference in zinc homeostasis and root growth (Bovet et al., 2006). In maize and soybeans, seed germination did not prove a good indicator of cadmium toxicity; while the roots of both these plants showed greater sensitivity to cadmium than shoots (Cao et al., 2007). In mungbean, postgermination mortality (instead of germination) was considered as an important cadmium-phytotoxicity effect (Ghani and Wahid, 2007). Comparison of three

ornamental species revealed that, on a dry weight basis, African marigold (*Tagetes erecta*) proved the most tolerant, scarlet sage (*Salvia splendens*) was the most sensitive, and sweet hibiscus (*Abelmoschus manihot*) showed high sensitivity with 50% inhibition in seed germination (Wang and Zhou, 2005). Significant differences have been reported for wheat genotypes in shoot cadmium concentration (Zhang et al., 2002). Cadmium is accumulated by many cereals, potatoes, vegetables, and fruits, and humans get at least 70% of it from plant food (Jackson and Alloway, 1991; Wagner, 1993).

Greater final economic yield is an index of metal tolerance by tolerant plant species. Studies show that cadmium applied to green gram plants at the rate of 24 mg/kg soil diminished the seed yield by 40%, while 50% diminution in nodule leghaemoglobin protein occurred at 12 mg/kg soil. This led to a substantial diminution of total grain protein (Wani et al., 2007).

In summary, although differences exist, most crop species and varieties are sensitive to cadmium stress. The cadmium toxicity effects are evident in terms of injury symptoms on the above-ground parts, reduced growth, and yield.

3.2 Anatomical and Developmental Responses

As an initial target, roots show wide-ranging responses to excesses of cadmium (Wojcik and Tukiendorf, 2005). Histolocalization experiments involving transmission electron microscopy (TEM) indicate the presence of cadmium deposits in the vacuoles of the exodermal cells in *Phragmites australis*. This shows that roots, as a tolerance mechanism, store the cadmium in the vacuole to protect the cytoplasm from its adverse effects (Ederli et al., 2004). Suzuki (2005) reported that cadmium causes cell death in the elongation zone of *Arabidopsis* roots. During incubation of the roots for two weeks in a sublethal level of cadmium, cells became deformed with irregularly thickened walls. These cells exhibited the accumulation of some chemical in the endodermis, pericycle, and cambium. Although a clear mechanism causing such changes could not be ascertained, it seemed that cadmium restricted the mitosis of the root cells (Vernoux et al., 2000). In addition to causing anatomical changes, cadmium alters the developmental phenomena at both the cellular and tissue levels. It induced the production of root hairs 2–4 mm behind the root tip, caused premature development of the root, hastened xylogenesis, and ultimately the production of shortened root, primarily by elevated hydrogen peroxide production and peroxidase activity in the early metaxylem and vascular bundles (Durcekova et al., 2007).

Detrimental effects of cadmium have also been reported in the cellular ultrastructures. Applied cadmium led to the disorganization of the chloroplast ultrastructure with an increase in the plastoglobulii and formation of vesicles in the vacuole. It caused the senescence of peroxisomes and induced a metabolic transition from peroxisomes to glyoxysomes (McCarthy et al., 2001).

In short, higher levels of cadmium have substantial influence in producing anatomical and developmental changes in the cells and tissues, including the disruption of organelles structure.

3.3 Cell and Tissue Localization

Having been taken up by the root and translocated to various cells and tissues within the plant, cadmium concentrates there and causes injury. Cadmium-tolerant tobacco species (*Nicotiana rustica*) indicated greater labeled cadmium (^{109}Cd) content in the roost than the leaves, the major part of which was stored in the distal part as a tolerance strategy (Bovet et al., 2006). Studies employing the use of analytical electron microscopy, x-ray spectromicroscopy and energy-dispersive x-ray microanalysis revealed the localization of cadmium in vascular bundles and linked to the cell wall of the S-ligands and pericycle in the root of *Arabidopsis thaliana*. However, leaf trichomes represented major compartments of cadmium accumulation (Wojcik and Tukiendorf, 2004; Isaure et al., 2006). Although in *Phragmites australis* roots a high dose of cadmium did not reveal any ultrastructural changes, histochemical localization exhibited the deposition of cadmium in the parenchyma cells below the exodermis (Ederli et al., 2004). However, in a study on the ultrastructure of *Arabidopsis thaliana* roots using energy-dispersive x-ray microanalysis, cadmium was found to deposit with phosphorus in the apoplast (Cd/P) and sulfur in the symplast (Cd/S), suggesting its precipitation with phytochelatins.

In endodermis, cadmium was sequestered as fine granular deposits in the cytoplasm (symplast). The passage cells appeared to play a role in the cadmium transport from pericycle to the stele for its entry again into the apoplast. In the leaves, the cadmium was detected in the tracheids but not the mesophyll cells. This indicated cadmium retranslocation from the shoot to the root (Van Belleghem et al., 2007). In the willow (*Salix viminalis*), major cadmium deposition took place in a pectin-rich layer of collenchyma cell walls in the veins, accelerated senescence of the mesophyll cells in the leaf blade, and caused tannin-plugging and necrosis in the leaf edges surrounding the mesophyll and upper epidermis (Vollenweider et al., 2006).

Many studies show that roots, because they are directly exposed, accumulate greater quantities of cadmium. In *Phaseolus vulgaris*, a greater cadmium accumulation in the root had no marked effect on the plastid ultrastructure. Younger leaves, compared to primary leaves, indicated a greater disruption of chloroplast structure and function (Barcelo et al., 1988). This indicated that when cadmium is transported to the shoot, photosynthetic (mesophyll) cells are more prone to cadmium toxicity where it is deposited, causing oxidative damage and enhancing senescence (Baryla et al., 2001; Vollenweider et al., 2006; Wahid and Ghani, 2008). In summary, cadmium is accumulated and deposited both in shoot and root tissues, where it interferes with physiological phenomena, and disrupts cellular structures.

3.4 Physiological and Biochemical Responses

Cadmium has two major effects on plant systems: inactivation of macromolecules and cellular structures, and induction of oxidative stress (Stroinski, 1999). Decline in growth and yield with elevated levels of cadmium in growth media have been attributed to factors like reduced photosynthetic rate (Verma and Dubey, 2002).

Nonetheless, mechanisms of cadmium toxicity are still the subject of intensive research, as discussed in the next sections.

3.4.1 Photosynthesis

It has been reported that all aspects of photosynthesis, including light and dark reaction and assimilate partitioning, are sensitive to cadmium excesses. It disturbs the chloroplast metabolism, either by inhibiting chlorophyll biosynthesis, enhancing its degradation at the heme level, or hampering photochemical and carboxylation reactions (de Filippis and Zeigler, 1993; Vassilev et al., 2003, 2005) by affecting the activities of chloroplastic enzymes (Chugh and Sawhney, 1999). Although all photosynthetic enzymes are affected, the enzymes of light reactions are specifically influenced (Kupper et al., 2007).

Among the light reactions of photosynthesis, applied cadmium readily and pronouncedly affects photosystem-II activity over short exposure periods in *Thlaspi caerulescence* (Kupper et al., 2007), and both photosystem-I and II over long periods of exposure in peas (Chugh and Sawhney, 1999). A greater arrest in the photosystem-II activity compared to photosystem-I was associated with greater reactive oxygen species generation and diminished antioxidant activities in *Riccia* (Prasad et al., 2004). However, in two maize cultivars, the photosystem-II activity was declined due to oxidative damage but did not cease, although there was a considerable loss in the levels of chlorophylls and carotenoids (Ekmekci et al., 2008).

Applied cadmium reduced the carbonic anhydrase activity and photosynthetic pigments in the leaves, and nitrate reductase activity and carbohydrate content in the root (Hayat et al., 2007) and leaves (Mobin and Khan, 2007), although lower levels enhanced carotenoid content (Prasad et al., 2004). Photosynthesis in sunflowers is mainly altered by cadmium-induced oxidative damage to chloroplastic membranes due to hampered Rubisco activity that diminishes photochemical and nonphotochemical quenching, and quantum efficiency of photosystem-II and CO_2 assimilation (Di Cagno et al., 2001).

Among gas-exchange parameters, stomatal conductance and its indices, transpiration and net photosynthetic rate, are greatly affected by cadmium (Sanita di Toppi et al., 1999; Balakhnina et al., 2005; Wahid et al., 2008). Reduction in transpiration rate of cadmium-treated plants might be due to stomatal closure. Although cadmium declines the stomatal conductivity, a beneficial aspect of such an effect might be associated with a limited transport of cadmium with reduced transpirational flow (Bindhu and Bera, 2001; Wahid et al., 2007b).

In summary, cadmium affects all the aspects of photosynthesis—light reactions of photosynthesis, particularly photosystem-II activity, and enzymes of dark reactions are specific targets of cadmium toxicity.

3.4.2 Water and Nutrient Relations

As a result of cadmium effects on root structure and functions, hampered water and nutrient status of plants is an immediate response. Available evidence suggests that

cadmium permeates the cytosol through calcium channels on the plasmalemma and changes the cell-water relationship (Perfus-Barbeoch et al., 2002; Teresa Milone et al., 2003). Acquisition of essential nutrients in appropriate amounts is important to plant growth, since they constitute either structural or functional components of cells (Epstein and Bloom, 2005).

Elevated cadmium levels substantially influence plant mineral nutrition, and a negative correlation has been reported for the uptake and distribution of various macro- and micronutrients in various plant parts (Shukla et al., 2003; Drazic et al., 2004; Adhikari et al., 2006; Ghnaya et al., 2007; Wahid et al., 2008). Cadmium-induced leaf chlorosis appears due to the antagonistic effect of cadmium on the uptake of iron, phosphorus, manganese, zinc, and copper, causing their deficiencies particularly in the cadmium-sensitive varieties (Alcantara et al., 1994; Epstein and Bloom, 2005; Chen et al., 2007a). It appears that the same metal transporters are employed for cadmium as for other metal ions (Sharma et al. 2004). Root membrane transporters involved in the uptake of potassium, calcium, and magnesium are the first targets of cadmium toxicity (Mengel et al., 2001). In cadmium-tolerant but non-hyperaccumulator *Matricaria chamomilla*, plants low levels of cadmium promoted potassium uptake, but higher levels stimulated potassium leakage from the root (Kovacik et al., 2006). This implied that changes in both water and nutritional relations are primarily due to altered root structure and functions.

3.4.3 Antioxidants and Other Enzyme Functions

Enzymes, as biochemical catalysts, are vital for metabolic functions in the cells. Like other metals, cadmium has a well-marked inhibitory effect on the activities of enzymes. Applied cadmium enhances the activities of glyoxylate cycle enzymes in the pea leaf peroxysomes, as well as activities of leucine-aminopeptidase and endopeptidase isozymes, which displayed senescence symptoms on the leaves (McCarthy et al., 2001). It reduces ATPase activity of the plasma membrane fraction of roots, leading to hampered transport and transfer processes at the root surface (Fodor et al., 1995; Obata and Umebayashi, 1997; Hall, 2002; Astolfi et al., 2005). In the root, nodules, and leaves of soybeans, cadmium completely inhibited the δ-aminolevunic acid dehydratase activity and led to a build up of δ-aminolevunic acid levels in these parts.

Exposure of sunflower plants to cadmium levels enhanced the arginine decarboxylase and ornithine decarboxylase activities, which enhanced the level of putrescine and spermine and diminished spermine and proline contents (Groppa et al., 2007). This accumulation resulted in the enhanced thiobarbituric acid reactive substances (TBARS) and diminished expression and activities of antioxidants, although S-adenosyl-L-methionine played a protective role against δ-aminolevunic acid induced oxidative damage (Leon et al., 2002; Mediouni et al., 2006; Noriega et al., 2007). Cadmium produces alterations in the functions of membranes by altering their lipid composition (Ouariti et al., 1997) and making them more permeable to solute leakage (Azevedo et al., 2005a). Studies suggest that such changes occur due to cadmium-produced oxidative damage to the membrane lipids due to the

production of free radical or diminished antioxidant activities (Foranzier et al., 2002; Cho and Seo, 2004). The senescence observed in soybean nodules treated with cadmium has also been attributed to the oxidative damage (Balestrasse et al., 2004).

Cadmium is assumed to be involved directly or indirectly in the formation of free radicals, thereby causing oxidative stress (Fig. 2). Higher concentrations of cadmium in the cytosol leads to the generation of reactive oxygen species in plants (Benavides et al., 2005; Smeets et al., 2005). A balance between the steady-state levels of different reactive oxygen species are determined by the interplay between different reactive oxygen species-producing and scavenging mechanisms. The physiological condition of the plant and the integration of different environmental, developmental and biochemical stimuli are important in this regard (Asada and Takahashi, 1987; Asada, 1999; Polle, 2001). Induction of antioxidants is a protective response against oxidative damage (Schützendübel et al., 2001; Sandalio et al., 2001). A variety of antioxidants, including superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and thioredoxin are the peroxiredoxin family of proteins (Bowler et al., 1992; Asada, 1999; Mittler, 2002). These protein antioxidants are supplemented with nonprotein scavengers, including intracellular ascorbate and glutathione. (Noctor and Foyer, 1998) to scavenge the reactive oxygen species, including superoxide and hydrogen peroxide, which enhance membrane permeability (Mobin and Khan, 2007). In *Bacopa monnieri* and potato tubers, the level of glutathione changes due to declined glutathione reductase activity (Stroinski et al., 1999; Mishra et al., 2006) or superoxide dismutase activity in *Brassica juncea* (Mobin and Khan, 2007). In maize, on the other hand, there was no change in the superoxide dismutase, ascorbate peroxidases, or glutathione reductase activities, but there was an increase in peroxidase activity under cadmium stress.

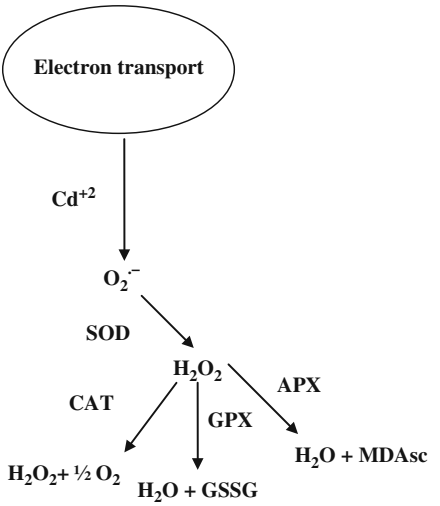

Fig. 2 Generation of reactive oxygen species by cadmium, and their scavenging mechanism by antioxidants (Modified from Pinto et al., 2003)

4 Cadmium Tolerance and Detoxification Mechanisms

When present at supraoptimal levels in the soil, cadmium is inevitably taken up by the roots and damages various plant organs and tissues (Sections 3.1. and 3.2.). Chemical and biological metal-binding capacity of the soil can reduce the uptake of cadmium. Plants can synthesize many metabolites, including phytochelatins, metallothioneins, and organic acids, which can bind and inactivate cadmium (Costa and Morel, 1993; Sanita di Toppi et al., 1999; Rauser, 1999; Schat et al., 2002; Clemens and Simm, 2003). However, their induction and synthesis demand some lag period, while the plant still experiences the toxic effects of metals (Wahid, 2008). Therefore, plant tolerance to metal toxicity cannot solely be attributed to the formation of complexes with the chelating metabolites. Phytohormones are also assumed to play an important role in the adaptation of plants to metal toxicity (Prasad, 1995). In view of this, cadmium tolerance in plants can be related to mechanisms occurring in soils and whole plants—cellular, physiological, and biochemical.

4.1 Soil Mechanisms

There are various mechanisms through which cadmium can be detoxified in the soil. Cadmium immobilization, involving fixation by solidification or stabilization through physical, chemical, or biological means, can prevent its migration into the groundwater (Lothenbach et al., 1999). Soil washing is another promising technique that involves the transfer of heavy metals into a wash solution either by desorption or solubilization (Semer and Reddy, 1996). Organic ligands such as organic acids and amino acids in soil may bind and chelate the cadmium and other heavy metals in the soil, thereby making them unavailable for soil microflora or absorption by the root (Mengel et al., 2001; Collins et al., 2003). Heavy metal ions, even under soil conditions of low pH or low organic matter, can quite often bind to soil particles in significant amounts. This binding affinity of cations also restricts cation movement into the xylem of vascular plants. A general solution to this problem is chelation, which is generally understood as a process of cation binding to a compound, resulting in a neutrally-charged complex that can move more freely via a variety of substrates.

Bioremediation in the rhizosphere may be another important strategy in coping with metal toxicity. High organic carbon and microflora of soil encourage degradation of organic chemicals in the soil. Available evidence shows that arbuscular mycorrhizal fungi facilitate cadmium uptake by bean and maize up to 41%, but this varies with soil pH and cadmium concentration (Guo et al., 1996). In a study, Heggo et al. (1990) found that arbuscular mycorrhizal fungi increased cadmium uptake in soybeans when the soil cadmium concentration was low, and vice versa. Although the mechanisms behind these phenomena are elusive, the arbuscular mycorrhizal fungi appear to offer potential for phytoextraction.

4.2 Whole Plant Mechanisms

Cadmium accumulates in many cereals, potatoes, vegetables, and fruits (Wagner, 1993; Jamali et al., 2007). Therefore, the tolerance of crops to cadmium toxicity may be related to their ability to absorb and accumulate cadmium in various parts (Leon et al., 2002). Difference in grain accumulation of two near-isogenic lines of durum wheat was due to diminished capacity for transport from root to shoot of the low cadmium-accumulating isoline. Furthermore, root phytochelatin synthesis had no influence on the shoot accumulation of cadmium in both the isolines (Hart et al., 2006). Application of higher levels of cadmium induced changes in stomatal openings and closings, and deposition of wax on both the leaf surfaces (Rai et al., 2005).

Normally, cadmium ions are retained in the root, and only small amounts are transported to the shoot (Cataldo, 1981). *Matricaria chamomilla* showed seven- to eleven-fold higher accumulations of cadmium in the roots. However, it was not classified as a hyper-accumulator and was found unsuitable for phytoremediation (Kovacik et al., 2006). On the contrary, Moral et al. (1994) reported that cadmium was easily transported to the aerial parts of tomato plants but was not detected in the fruit. In general, the content of cadmium in different plants declined in the following order: roots > stems > leaves > fruits > seeds (Blum, 1997). In cotton, the cadmium accumulation was in the following order: root > petiole > xylem > fruiting branch, leaf > phloem in vegetative organs and seed coats, seed nut > boll shell > fiber in reproductive organs (Wu et al., 2004). Exposure of potatoes to radioactive cadmium (^{109}Cd) showed that basal roots retained a greater proportion of the absorbed cadmium, while tubers and associated stolons contributed only a minor fraction at the vegetative stage. Short-term experiments using foliar cadmium application showed that newly absorbed cadmium was rapidly sequestered by the stem, which acted as a transitional storage pool. During long-term study, the leaves constituted a major pool, indicating redistribution of the absorbed cadmium within the plant body (Reid et al., 2003). Studies at reproductive stage of barley revealed that cadmium was partitioned into various parts of the spike and the partitioning was in the order: awn > stem > grain > rachis > glum. However, awn-removal and stem girdling decreased the amount of cadmium in the grain, implying their role in controlling cadmium translocation to grains (Chen et al., 2007b).

In summary, as a tolerance mechanism, plants partition cadmium in various parts, including roots, above-ground vegetation, and reproductive parts.

4.3 Cellular Mechanisms

To detoxify cadmium effects and protect the physiologically active sites from cadmium damage, plants have four general cellular strategies: (1) metal binds to the cell wall, (2) transport across cell membranes is reduced, (3) compartmentalization, and (4) chelation (Prasad, 1995; Dan et al., 2002; Clemens, 2006). Most plants utilize one or more of these strategies.

4.3.1 Cell Wall Binding

Preventing cadmium ions from entering the cytosol by the plant cell walls could theoretically represent the best detoxification mechanism (Ernst et al., 1992; Blaudez et al., 2000). Cell walls of the root can act as a first barrier against cadmium stress in immobilizing excesses of cadmium (Nishizono et al., 1989). Available evidence suggests that cadmium binds to the secondary wall and middle lamellae in maize roots (Kahn et al., 1984). On the other hand, in bush bean, cadmium was mainly bound to pectic sites and hystidyl groups of the cell wall in roots and leaves (Leita et al., 1996). In white lupin, the cell wall was found to retain up to 47% of the absorbed cadmium in leaves, 51% in stems, and 42% in the root, and this accumulation was related well to enhanced phytochelatins synthesis, particularly in the roots. This implied that cell-wall binding is a major detoxification mechanism (Vazquez et al., 2006).

4.3.2 Reduced Transport

Although not fully established, the transport of cations across cell membranes is achieved by transporters. Molecular studies led to the cloning of copper, zinc, and iron transporters from *Arabidopsis thaliana* (Salt et al., 1998). Blocking the transcription of gene coding for transporters could enhance the tolerance of plants to heavy metals (Prasad, 1995). Subsequent to metal uptake into the root symplasm, the movement of metals from roots into the xylem is governed by: (1) sequestration of metals inside root cells, (2) symplastic transport into the stele, and (3) release into the xylem (Clemens et al., 2002). During transport, cadmium is transferred to phloem and rapidly distributed throughout the plant (Reid et al., 2003).

Various transporters are involved in heavy metal resistance; the most important of these are a family of ATP-binding cassette transporters reported from bacteria, yeast, plants, and animals that transport a wide variety of materials across the cellular membranes (Higgins, 1992). A p-type pump, ZntA, from *Escherichia coli* (Sharma et al., 2000), and two ATP-binding cassette transporters from yeast (Ortiz et al., 1995; Li et al., 1997) make complexes of cadmium with phytochelatins, and transport into the vacuole. Among plant ATP-binding cassette transporters, only AtMRP3 (Bovet et al., 2003) and AtATM3 (Kim et al., 2006) have been reported to sequester cadmium into the vacuole. Bovet et al. (2003) reported the greater synthesis of four putative sequences coding for *ATMRPs* genes after cadmium treatment in *Arabidopsis thaliana*. Furthermore, phytochelatins, glutathionein, and oxidative stress were consistent with these transporters' gene expression. Another ATP-binding cassette transporter is AtPDR8, which when overexpressed in the transgenic *Arabidopsis thaliana* plants, conferred resistance against cadmium. Use of radioactive cadmium provided evidence that AtPDR8 was an efflux pump of cadmium ions or cadmium conjugates at the plasmalemma of *Arabidopsis thaliana* (Kim et al., 2007).

4.3.3 Compartmentalization

Free and complexed cadmium is sequestered in the vacuole of root cells in most species. It is actively transported from the cytosol into the vacuole across the tonoplast via proton/cadmium (H^+/Cd^{2+}) antiport or an ATP-dependent phytochelatin transporter. A gene coding for a phytochelatin-transporter (*hmt1*) was isolated in yeast (Salt and Wagner, 1993). When overexpressed in plants, this gene allowed for enhanced production of phytochelatin (PC) transporters, which boosted the ability of a plant to sequester PC-cadmium complexes in the vacuole (Ortiz et al., 1995). One recurrent mechanism for detoxifying heavy metals in plants and other organisms is the chelation of metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand-metal complex. Vacuolar compartmentalization prevents the free movement of cadmium in the cytosol and forces it into a limited area (Sanita di Toppi and Gabbrielli, 1999). Several studies suggest that the vacuole is the main accumulation site for a number of heavy metals, including zinc and cadmium (Ernst et al., 1992; Benavides et al., 2005). Several metal-binding ligands, including organic acids, amino acids, and polypeptides, have been recognized in plants (Rauser, 1999).

4.3.4 Chelation

Several chelators are known to perform the function of chelation. Among the natural chelators, phytochelatins and metallothioneins are the main metal-chelating proteins expressed in plants. Of these, phytochelatins are small polypeptides produced in plants by enzymes, which are expressed in response to heavy metals or oxyanions (Prasad, 1995; Clemens, 2006). A majority of the reports show that phytochelatins constitute a major cadmium-detoxification system in plants. They are accumulated in both shoot and root; however, their predominant synthesis in either tissue depends upon the tendency of the species to biosynthesize them (Clemens and Simm, 2003; Rauser, 2003; Clemens, 2006). Use of buthionine sulfoximine—an inhibitor of glutathione synthesis—revealed the formation of cadmium-phytochelatins complexes and cadmium detoxification in *Arabidopsis thaliana* (Wojcik and Tukiendorf, 2004). Alternatively, the role of phytochelatins in the sequestration of cadmium by the transporters at plasmalemma and tonoplast is a likely mechanism in plants (Gadapati and Macfie, 2006). In *Schizosaccharomyces pombe*, however, it is shown that absorbed sulfate after being assimilated with cysteine is incorporated into phytochelatins that bind cadmium and are transported to the vacuole by an ATP-binding cassette transporter (Hmt1) as a low molecular-weight complex, which with the availability of more sulfur-containing compounds is converted to a high molecular-weight complex (Fig. 3). Studies on maize roots show that, over a short-term cadmium-stress period, low molecular-weight cadmium-phytochelatin complexes predominated in the apices, with high molecular-weight ones in the mature zone of maize roots. However, this binding of cadmium is in a dynamic state and depends upon the treatment and its amount in the tissue (Rauser, 2003).

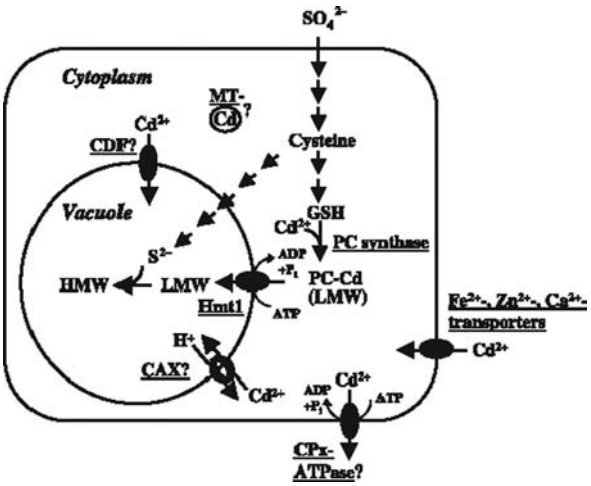

Fig. 3 Possible mechanisms for the detoxification of Cd by phytochelatins (PC). Abbreviations: GSH (glutathione), LMW (low molecular weight complex), HMW (high molecular weight complex), CAX (protein mediating pH dependent high affinity for Ca^{2+} uptake through tonoplast), CDF (cation diffusion facilitator protein), Hmt1 (ABC-type transporter at tonoplast for transport of PC-Cd LMW). Reproduced with permission from Blackwell

Studies show that phytochelatin accumulation has a protective role in physiological phenomena against cadmium toxicity. In addition to phytochelatin involvement in photosynthetic processes, their enhanced accumulation has been suggested as phytoremediation through its antioxidative mechanism (Mishra et al., 2006). A greater effect of cadmium on the chlorophyll fluorescence parameters of maize leaf segments was considerably offset by cadmium-induced phytochelatins and thiol(-SH)-specific molar ratios (Drazkiewicz et al., 2003). Contrary to this, it has been shown that phytochelatin-accumulation has a negative correlation with chlorophyll content in *Brassica juncea* and *B. napus*. Although phytochelatins appear to be mainly responsible for modulating a cadmium-phytotoxicity response of plants, a recent study suggests that in the cadmium hyper-accumulating *Sedum alfredii* plant, cadmium leads to a greater accumulation of glutathione (GSH) rather than phytochelatins—the activity of which is consistent with GSH accumulation (Sun et al., 2007).

Unlike phytochelatins, metallothioneins are found both in animals as well as plants. They are gene-encoded polypeptides with an apparent molecular mass of 8–14 kDa (Robinson et al., 1993), and thought to be aggregates of phytochelatins (Prasad, 1995). In the plant kingdom, metallothionein-like proteins are reported in a number of plant species (Wojcik and Tukiendorf, 2005; Clemens, 2006). Metallothioneins behave similar to phytochelatins, and metal complexation is often shared by both, as observed in *Datura* and maize (Rivai, et al., 1990). Introduction of mammalian metallothionein into *Brassica campestris, B. napus*, and *Nicotiana tabacum* conferred cadmium tolerance by promoting complexation and minimizing translocation of cadmium via xylem to shoots (Maiti et al., 1989).

Cysteine is an important amino acid, which is a precursor and an integral component for the biosynthesis of glutathione, phytochelatins, and thiolate peptides involved in the detoxification and transport of cadmium and many other

heavy metals to vacuoles (Cobbett, 2000). Therefore, enhanced synthesis of cysteine is imperative for efficient heavy-metal detoxification. Genetic transformation in *Arabidopsis thaliana* using *Atcys-3A* construct expression cytosolic O-acetylserine(thiol)lyase (OASTL) indicated that the tranformant in the presence of cadmium indicated significantly higher cysteine biosynthesis than the wild type, which was responsible for enhanced tolerance and accumulation of cadmium in the leaves (Dominguez-Soils et al., 2004).

In summary, the synthesis of chelating proteins constitutes a major cadmium-detoxification mechanism. The major roles of phytochelatins remain the protection of photosynthetic machinery and membrane structure from cadmium damage.

4.3.5 Complexation

Organic acids have great potential to form complex with heavy metals. In this regard, both citric acid and malic acid have been shown to complex heavy metals in plant roots (Benavides et al., 2005). After the loss of H^+, each acid contains a COO^- group, which binds to the cation. Plants secrete acids that aid in the uptake of nonbioavailable metals (Larsen et al., 1998). These acids protect cellular function when the acid-cadmium complex is brought into the root. Citric acid metal complexes have been shown to translocate via the xylem (Senden et al., 1990). Genetic alteration of plants for producing higher levels of endogenous citric acid or malic acid may lead to enhanced phytoextraction of the metals, including cadmium.

4.4 Physiological Mechanisms

Heavy metal accumulation influences various metabolic functions in plants. However, plants show some adaptive physiological mechanisms for cadmium tolerance as detailed in the following section.

4.4.1 Water and Nutrient Transport

Cadmium has been shown to interfere with the transport of water, and uptake and the distribution of several macro- and micronutrients in plant roots (Gussarson et al., 1996; Hernandez et al., 1996; Das et al., 1997). Some evidence suggests that under excessive transpiration rates, cadmium permeates the cytosol through calcium channels on the plasmalemma, and hampers the cell water status (Perfus-Barbeoch et al., 2002; Teresa Milone et al., 2003).

Data suggest that cadmium can alter the uptake of minerals by plants either by reducing the availability of minerals in the soil or through a reduced population of soil microbes (McGrath et al., 2001). Cadmium has been shown to interfere with the transport and distribution of several macro- and micronutrients in plants roots (Gussarson et al., 1996; Hernandez et al., 1996; Das et al., 1997). Ederli et al. (2004) showed that *Phragmites australis* roots could tolerate higher levels of cadmium by accumulating in parenchymatous cells below the endodermis. Substantial declines

in nitrate reductase activity, nitrogen fixation, and primary ammonia assimilation in nodules were found in legumes due to cadmium application (Balestrasse et al., 2004). In the cadmium-hyperaccumulator *Arabidopsis halleri*, the use of radioisotopes indicted that cadmium and zinc employed the same transporter, but that cadmium detoxification did not follow the zinc-detoxification mechanism (Zhao et al., 2006).

4.4.2 Photosynthesis and Assimilate Partitioning

Photosynthesis represents a key metabolic pathway in plants in the production of energy-rich organic compounds. The assimilation of these high-energy compounds in growth phenomena requires a balanced supply of reducing powers. The maintenance and activity of photosystem-II is of vital importance in the regulation of electron transport. An increasing number of studies show that photosystem-II is highly sensitive to stressful conditions, including that of cadmium toxicity (Chugh and Sawhney, 1999; Prasad et al., 2004; Ekmekci et al., 2008); however, understanding the precise mechanisms of cadmium toxicity on various aspects of photosynthesis is still the subject of intensive research. Pulse-chase labeling experiments employing [^{35}S]methionine indicated a great effect of cadmium on the synthesis, degradation, and assembly of D1 protein, which appeared to be due to an unknown primary effect of cadmium on the photosystem-II apparatus (Geiken et al., 1998). In wheat, an efficient sulfur assimilation and antioxidative system was helpful in protecting the photosynthetic ability and maintaining high-yield potential under cadmium stress (Khan et al., 2007).

4.4.3 Membrane Damage and Antioxidative Defense

As discussed earlier (Section 3.5.3.), one of the most pronounced effects of cadmium toxicity is the production of reactive oxygen species, which leads to peroxidation of membrane lipids and disrupts the normal membrane functions. Among various reactive oxygen species, generation of hydrogen peroxide is more damaging due to its relatively longer life (Foyer and Noctor, 2005). Mitochondria and peroxisomes of mesophyll and guard cells produce hydrogen peroxide catalyzed by the enzyme NADP-oxidase, which is localized on the tonoplast of the bundle sheath and plasma membrane of mesophyll cells, where it is involved in the production of activated oxygen and hydrogen peroxide (Hsu and Kao, 2004; Romero-Puertas et al., 2004). Tobacco cell death during exposure to cadmium chloride was accompanied by successive waves of reactive oxygen species generation, which differed in their nature and subcellular localization. These were (a) NADPH-oxidase dependent accumulation of hydrogen peroxide, (b) accumulation of activated oxygen in mitochondria, and (c) a wave of reactive oxygen species consisting of fatty acid hydroperoxide, which was concomitant with cell death (Garnier et al., 2006). This was confirmed from the fact that cell line gp3, impaired in NADPH-oxidase activity, was unable to accumulate hydrogen peroxide. Nevertheless, the cell death appeared to be due to cell poisoning by cadmium (Garnier et al., 2006). Thus, inhibition of the

activity of NADP-oxidase can reduce the content of NADP-oxidase. Treatment of detached rice leaves with diphenyleneiodonium chloride and imidazole, inhibitors of NADP-oxidase, prevented cadmium chloride-induced hydrogen peroxide production, suggesting that cadmium chloride toxicity was primarily due to generation of hydrogen peroxide (Hsu and Kao, 2007b).

Glutathione reductase (GR) is an important antioxidant enzyme that exists in various isoforms, is expressed in various tissues, and prevents oxidative damage to plants subjected to a variety of stresses including cadmium toxicity. In the roots (but not the shoots) of wheat, no change in mRNA and protein expression occurred, but occurrence of posttranslational modification was evident. These changes induced distinctive isoforms and up-regulated the GR activity as a defense mechanism against oxidative stress (Yannarelli et al., 2007).

4.4.4 Modulation of Hormonal Levels

Hormones play important roles in the adaptation of plants to stressful environments (Wahid et al., 2007a). Cadmium causes the senescence of cells via intricate mechanisms. It may lead to enhanced biosynthesis of ethylene and reactive oxygen species and a decline in nitric oxide, which promote cellular senescence. The synthesis of jasmonic acid and salicylic acid may regulate the cellular response to combat cadmium damage (Rodriguez-Serrano et al., 2006). Accumulation of abscisic acid and diminution of cytokinins has been reported in the plants treated with toxic metals (Poschernirieder et al., 1989; Prasad, 1995). In an attempt to elucidate the role of abscisic acid in cadmium tolerance, Shanti and Kumar (2002) noted that seed germination and seedling growth of abscisic acid-deficient and abscisic acid-insensitive mutants were comparable to wild-type plants of *Arabidopsis thaliana*, which suggested no mediatory role of abscisic acid in cadmium tolerance (Sharma and Kumar, 2002).

5 Mitigation of Cadmium Toxicity Effects

The toxic effects of cadmium are both acute and chronic. For sustainable crop production, it is imperative that strategies may be adopted to alleviate the effects of cadmium toxicity. In this regard, some research efforts have been undertaken that are summarized in Table 2 and briefly described in the next section.

Ethylene diamine tetra acetic acid is a synthetic chelator, and has been shown to substantially lower the cation exchange capacity (CEC) of soil. It has been used frequently since the 1950s to alleviate iron deficiency and improve phytoextraction of metal contaminants, such as lead, from soil (Jorgenson, 1993). Invariably, a sudden rise in bioavailable metals with the applied ethylene diamine tetra acetic acid may be fatal to plants. This can be overcome by growing plants up to advanced stages and then adding ethylene diamine tetra acetic acid. In this way, the metal becomes more bioavailable and is taken up in large quantities by the plant for a short time before the plant dies. Thus, large amounts of metal can be extracted from soil and

Table 2 Effectiveness of some organic and inorganic sources on the alleviation of cadmium's pytotoxic effects. The ameliorative action of various sources is quite diversified and depends upon the plant species

Plant species	Amelioration source	Mode of application	Effective concentration	Major effect	Approximate improvement	Reference
Rice (*Oryza sativa*)	Salicylic acid	Seed presoaking	100 μM for 16 h	Induction of antioxidants	2–20%	Panda and Ratra (2007)
Soybean (*Glycine max*)	$Ca(OH)_2$	Sand culture	200 ppm	Non-availability of Cd	10–19%	Chaney et al. (1977)
Mustard (*Brassica juncea*)	Phosphate as KH_2PO_4	Soil culture	10 mg P/kg soil	Immobilization of Cd in soil	Up to 88%	Bolan et al. (2003)
Alfalfa (*Medicago sativa*)	Salicylic acid	Seed imbibition	10 μM for 3 h	Improved Mg and Ca status of shoot and root	22–40%	Drazic et al. (2006)
Rice	Polyamines (spermidine & spermine)	Pretreatment of detached leaves	5 mM for 6 h in dark	Reduction in H_2O_2 and MDA increase in ascorbic acid reduced glutathione production	4–43%	Hsu and Kao (2007b)
Kidney bean (*Phaseolus vulgaris*)	Lanthanum chloride	Foliar spray	10 mg/L	Improved membrane permeability, chlorophyll content, activities of catalase and peroxidase and reduced Cd uptake	3.5–37.5%	Xiaohua and Qing (2006)
Corn (*Zea mays*)	As above	As above	20 mg/L	As above	2.1–14.5%	As above
Barley (*Hordeum vulgare*)	Fe (Fe tartarate)	Nutrient solution	250 μM	Antagonistic effect of Fe on Cd uptake	Significant	Sharma et al. (2004)

this strategy is referred to as "chelator-assisted phytoextraction" (Salt et al., 1998). A related compound, ethylene glycol tetra-acetic acid, has also been shown to be quite effective in enhancing cadmium bioavailability for phytoextraction (Blaylock et al., 1997).

The genotypic flexibility of species and cultivars for cadmium accumulation and tolerance (Section 3.1.) can be exploited to breed and select desired materials. Breeding for cadmium tolerance is a long-term and cost-intensive venture, while selection of the desired material can be made in a short span of time. Exogenous use of osmoprotectants and other organic and inorganic compounds, including monosaccharides, quaternary ammonium compounds, amino acids, organic acids, ascorbate, polyamines, and growth regulators, etc., is an effective approach to combatting cadmium toxicity. Exogenous application of glucose and reduced forms of glutathione were effective in enhancing photosynthetic oxygen evolution and reducing the tissue content of cadmium (El-Naggar and El-Sheekh, 1998). Soybean plants treated with sodium naphthenate diminished 40% of intracellular cadmium in roots, stems, and leaves (on average), resulting in alleviation of cadmium toxicity with the activity of nitrate reductase and photosynthetic pigments (Ewais, 1997; Kevresan et al., 2004). Likewise, exogenous application of ascorbate was beneficial in enhancing the activities of antioxidative enzymes and alleviating oxidative damage and improving light harvesting under cadmium stress in barley (Wu and Zhang, 2004).

Plant-growth regulators function as signal molecules under a variety of stresses (Taiz and Zeiger, 2006). Studies show that use of salicylic acid, either as a presowing seed treatment in alfalfa (Drazic et al., 2006) and rice (Panda and Patra, 2007), or as its supplementation to soybean seedlings in nutrient solutions (Drazic and Mihailovic, 2005), was effective in lowering cadmium-induced oxidative damage in rice leaves and improving the growth and homeostasis of potassium, magnesium, and calcium. In another study, barley grains soaked with salicylic acid exhibited little effect in the absence of cadmium, but promoted root and shoot length and fresh and dry weight, and inhibited lipid peroxidation in roots in the presence of cadmium (Metwally et al., 2003). Polyamines, mainly spermidine and spermine, alleviated the cadmium toxicity on detached rice leaves by curtailing the cadmium uptake and production of hydrogen peroxide and malondialdehyde (Hsu and Kao, 2007a). *Brassica juncea* fed with 0.01 μM 28-homobrassinolide in nutrient solution and subsequently treated with cadmium levels enhanced the activities of antioxidants both in the shoot and root and contents of proline in the aerial parts. This suggested the role of 28-homobrassinolide in inducing cadmium tolerance in this plant species (Hayat et al., 2007).

Although cadmium has an antagonistic effect on the acquisition and transport of mineral nutrients (Section 4.4.1.), available evidence suggests that exogenous supply of some nutrients and inorganic sources can alleviate the cadmium phytotoxicity. However, the mitigatory roles of various nutrients may be different. For instance, lime (calcium hydroxide) has long been known to prevent the phytotoxic effect of cadmium (Chaney et al., 1977). Supplementing with 30 mM of calcium in the nutrient solution restored root elongation of *Arabidopsis* by diminishing up to 70% of the cadmium content of the seedlings compared to that treated

with cadmium alone. This happened because calcium was able to compete and alleviates cadmium toxicity through competition for influx (Suzuki, 2005). Among other nutrients, application of phosphorus mitigated the phytotoxic effect of cadmium and promoted dry matter yield of mung bean (Panwar et al., 1999) and soybean (Arao and Ishikawa, 2006). Similar effects of zinc were also reported in wheat and barley plants exposed to cadmium in vivo (Zhao et al., 2005; Chen et al., 2007a). In durum and bread wheat, necrotic patches developed on the leaf base and sheath due to cadmium toxicity were offset by the external supply of zinc (Koleli et al., 2004). Hassan et al. (2005) reported that the application of increased levels of sulfur to cadmium-stressed rice cultivars reduced the activity of superoxide dismutase and growth with a concomitant decline in malondialdehyde and tissue content of cadmium. The supply of magnesium in nutrient solution alleviated the cadmium toxicity by lessening chlorosis, the cadmium content of tissue, and enhancing potassium and zinc content in shoots and roots (Kashem and Kawai, 2007). Exogenously applied lanthanum was beneficial in improving root structure, chlorophyll content, and photosynthetic capacity and activities of antioxidants, while reducing membrane permeability and malondialdehyde in cadmium-treated kidney beans and corn (Xiaohua and Qing, 2006).

In short, application of some synthetic metal chelators and organic acids is important in alleviating the cadmium phytotoxicity in soils. For plants, exogenous applications of nutrients, polyamines, and plant-growth regulators can induce cadmium tolerance in plants by improving mineral homeostasis, antioxidant defense, and membrane properties.

6 Conclusion

Cadmium is a great threat to soil processes, plant growth, and productivity around the globe. Plant responses to cadmium toxicity are elicited at morphological, physiological, and biochemical levels. These include stunted growth, changes in the structure and function of organelles, diminished photosynthesis, effects on the membrane transporters, modulation of metabolic pathways and altered gene expression. Plants display a range of mechanisms to cope with the adverse effects of cadmium—the most important of these include reduced uptake from the soil, binding of the absorbed cadmium to cell walls, storage intcellular compartments, and detoxification by metal chelating and complexing, such as organic acids, phytochelatins, and metallothioneins. Cadmium toxicity can be overcome by the use of metal chelators in the soil, thereby reducing its bioavailability to plants. Breeding and selection of plants showing reduced ability to accumulate cadmium in the cells and tissues, and/or its efficient binding, complexation, and compartmentation, seed and foliar application of osmoprotectants, mineral nutrients, and plant-growth regulators are among the important strategies for mitigating cadmium toxicity on plants. Concerted research efforts on finding novel compounds with the ability to bind and inactivate cadmium, earmarking plant species capable of effectively binding or excluding metal at root levels with their lowered tendency to transport to shoot,

and partitioning to grain are desirable. This will be beneficial for more economical utilization of such plant species, and their sustainable production in marginally cadmium-contaminated soils.

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